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ACTIVE TRANSPORT AND SECRETION

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PREFACE

This volume contains the papers read at a Symposium of the Society for Experimental Biology which was held at Bangor in July 1953. It is the eighth of an annual series of Symposium Reports. The Symposium for 1954 will be held at Leeds, on Fibrous Proteins.

In the present Symposium the first three papers are introductory in character. These are followed by four papers on water movements and four papers on active transport phenomena in red blood cells, yeast and bacteria. The next group of five papers are concerned with active transport of ions in plants, and are followed by four papers on active transport of ions in animal cells. The remaining three papers are concerned with active movements of proteins and fats, and with mechanisms of active transport.

The papers presented here should be considered in relation to a number of recent reviews, notably by Conway (1953) (*Biochemistry of Gastric Secretion.* Springfield: Thomas), Hodgkin (1951) (*Biol. Rev.* **26**, 339), Brown (1952) (*Int. Rev. Cytol.* **1**, 107), Goldacre (1952) (*Int. Rev. Cytol.* **1**, 135) and Sutcliffe (1953) (*Int. Rev. Cytol.* **2**, 179).

The Society is deeply indebted to the British Council, the Rockefeller Foundation and to Imperial Chemical Industries Ltd. for financial aid.

The Editors wish to thank the members of the Advisory Committee who assisted us in preparing the Symposium programme. We also wish to thank the Cambridge University Press for the kindness with which we were assisted in producing this report.

R. BROWN
J. F. DANIELLI
Symposium Editors
Society for Experimental Biology

17 *February* 1954

MOVEMENTS OF WATER AND ELECTROLYTES IN INVERTEBRATES

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I. INTRODUCTION

When I was asked to give this introductory paper it was suggested to me that I should first and foremost present the background against which modern developments can be seen in perspective. It would be neither practicable nor desirable to attempt a review of all the material available; instead, what I shall do is to trace the development of ideas in the subject, to consider why some lines of approach have prospered more than others, why some questions have been answered and others left unasked, to show how the existence of active transport mechanisms has been recognized and to put forward some suggestions as to how they may have been evolved.

A few analyses of the body fluids of invertebrates were published during the latter part of the nineteenth century, and work of this type continued sporadically throughout the first two decades of the twentieth. At this stage these investigations were not inspired by any precise theory as to the nature of the body fluids and the ways in which their compositions were maintained; nevertheless, it became apparent that the body fluids of animals were in general not unlike sea water. Owing to the relative ease with which freezing-point measurements can be made our knowledge of the osmotic pressure of the body fluids began to advance rapidly. Experiments were carried out to test the effects of changes in the external medium upon the osmotic pressure of the body fluid. On the other hand, determination of the constituents of the body fluids was beset by technical difficulties and advance was slower. The development of the subject can be followed in the reviews which have appeared from time to time, notably those of Duval (1925), Schlieper (1930, 1935), Pantin (1931), Krogh (1939) and Beadle (1943). By the time the subject became of sufficient importance to merit review it had also acquired a philosophy which conveniently rationalized its ecological and physiological aspects—Claude Bernard's now famous pronouncement 'la fixité du milieu intérieur est la condition de la vie libre'. For the subject now under discussion Claude Bernard's pronouncement has the following special implication: primitive marine animals have in general no means of regulating the composition of their body fluids, and penetration into fresh water is only possible for animals which have evolved such means. Although the reviewers I have mentioned may have been mainly concerned with the physiological mechanisms whereby constancy is achieved, they have very obviously accepted Claude Bernard's proposition and incorporated it into the background of their ideas.

Let us then begin by considering a primitive marine animal having no control over the composition of its body fluid. When such an animal is placed in dilute sea water it swells, which may be interpreted as due to the inward diffusion of water. After some time in dilute sea water the volume of the animal returns to normal, which may be interpreted as the result of the relatively slower outward diffusion of salts through the general body surface. This, of course, is to look upon the animal as little more than a bag containing sea water. But even a primitive animal is generally something more than this. We have to consider that it has an alimentary canal, that it takes in food together with some sea water, and that it voids faeces which also have some admixture of fluid. It has an excretory organ from which urine is eliminated. Even if the body fluid is isotonic with sea water and the net exchange across the external surface is zero, the animal continually gains water, partly along with its food and partly as metabolic water produced by the oxidation of the food within the body, and loses water with its urine and faeces. There is thus a current of water continuously maintained through the body, upon which other movements, such as occur when the animal is placed in dilute sea water, are superimposed. The recovery of normal volume in dilute sea water, which we considered a moment ago, is not primarily due to leakage of salts and water through the general surface; it is due to an increased flow of urine, and it is via the excretory organ that most of the water and most of the salt leaves the body. There are in fact three principal regions of the body through which exchanges with the external medium can and do take place: (1) between the body fluid and the external medium at the surface of the body, (2) between the body fluid and the fluid in the gut, (3) between the body fluid and the urine in the excretory organ. All of these can be the sites of active transport mechanisms.

Next, let us consider what happens when this primitive marine animal evolves the ability to live in fresh water. This is a question which has been discussed at length by Beadle & Cragg (1940) and by Beadle (1943), and they have come to the conclusion that there are two stages in the process. I can most conveniently illustrate their thesis with examples drawn from the Crustacea (see Fig. 1). The spider crab *Maia* will serve to represent the primitive marine animal. It has virtually no powers of osmotic regulation, the osmotic pressure of its blood following that of the external medium over the whole of its viable range. The shore crab *Carcinus* shows some powers of osmotic regulation, being able to maintain the osmotic pressure of its blood above that of dilute sea water. It is commonly found in estuaries as well as in the sea but cannot maintain itself in fresh water. As an example of a crustacean fully adapted to fresh water we will take the crayfish *Astacus*. In this case the general level of the osmotic pressure of the blood is lower than in *Maia* and *Carcinus*, but it can be maintained at this level even in fresh water.

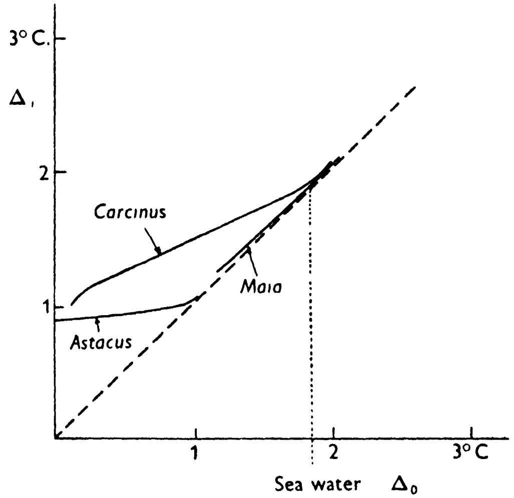

Fig. 1. Relation between the osmotic pressure of the blood, and the osmotic pressure of the external medium, for three crustaceans. *Maia* from Duval (1925), *Carcinus* from Duval (1925) and Schmidt-Nielsen (1941), *Astacus* from Herrmann (1931).

It might well have been imagined by the early investigators that these animals, when placed in dilute sea water, would follow what seems to us the obvious and logical course of pumping out the water which diffuses into them. But this does not seem to be the case. In a recent article, Robinson (1953)* has reviewed the evidence for the active transport of water in living systems, and as far as the aquatic invertebrate Metazoa are concerned the evidence is as yet circumstantial. It appears that these animals prefer to transport dissolved substances in such a way as to compensate for the passive movements of water under osmotic gradients.

According to Beadle & Cragg, in the first stage of the evolutionary process the animal develops the power of actively transporting salts from

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* I wish to thank Dr Robinson for allowing me to see his review in typescript before publication.

the external medium into the blood, to an extent which is sufficient to maintain the osmotic pressure of the blood significantly above that of the external medium. The excretory organ, however, does not back up the effort of the surface membranes. In *Carcinus* the urine is isotonic with the blood under all conditions, and beyond a certain point of dilution more salt is lost via the urine than can be gained by absorption through the surface. Ecologically this point lies for *Carcinus* some distance up the estuary, but definitely short of the river. But there is a crustacean which does in fact succeed in getting into rivers on this same inefficient physiological basis, and that is the Chinese mitten crab *Eriocheir*. As in *Carcinus*, so in *Eriocheir* the urine is isotonic with the blood under all conditions, but by sheer hard work, by absorbing salts from the river water at a great rate, this determined animal penetrates up rivers such as the Elbe for hundreds of miles, returning to the sea only for the purposes of breeding. For *Eriocheir* the ecological limit seems to lie not between brackish water and fresh water as for *Carcinus*, but between hard fresh water and soft fresh water; *Eriocheir* does not appear to be able to penetrate the softer waters of the Norwegian rivers (Schmidt-Nielsen, 1941).

Then in the second stage of the evolutionary process two things happen. First, the excretory organ becomes awakened to a proper sense of its responsibilities and produces hypotonic urine, thus conserving the salt content of the body; secondly, the general level of the osmotic pressure of the blood is lowered to about half that found in marine animals. This reduces the strain on the active transport mechanisms at the body surface and in the excretory organ. It also involves some readjustment of the salt and water balance between the tissues and the blood.

It emerges from this survey that the important sites of active transport lie in the general body surface—or specialized parts of it—and in the excretory organ. There is as yet very little evidence of active transport in the gut, at least as far as the digestive epithelium is concerned. We will therefore now proceed to further consideration of the surface membranes and of the excretory organs in aquatic invertebrates.

II. THE TRANSPORT OF IONS BY SURFACE MEMBRANES

The first demonstration that an animal can maintain the osmotic pressure of its blood by uptake of salts against a concentration gradient was given by Nagel (1934) for *Carcinus*. Having confirmed that *Carcinus* had the power of hypertonic regulation in brackish water and having demonstrated that the urine was always isotonic with the blood, Nagel carried out the following well-planned experiment. He took a number of crabs and allowed them to become adapted to a medium of considerable dilution. Some of the crabs were killed and measurements were made of the osmotic pressure and chloride content of their blood. The rest of the crabs were then placed in another medium, more concentrated than the first medium but less concentrated than the blood of the crabs which had become adapted to the first medium. After 24 hr. in the second medium the crabs were killed and their blood taken for analysis. Nagel's figures (Table 1) show that in the second lot of crabs both osmotic pressure and chloride concentration of the blood had increased. Since the body volume remained more or less constant the increase of osmotic pressure and chloride concentration could only be explained by the uptake of salts from the external medium against the concentration gradient. This uptake was not affected by blocking the mouth and Nagel assumed that it occurred at the gills.

Table 1. *Demonstration of active transport of chloride by* Carcinus

(From Nagel, 1934)

	External medium		Blood	
	Δ° C.	Cl (mg./ml.)	Δ° C.	Cl (mg./ml.)
I.	0·89	8·57	1·42	12·0
			1·20	11·9
			1·23	12·3
			1·42	13·0
			1·28	11·5
			Av. 1·31	12·1
II.	1·18	11·45	1·50	14·0
			1·52	14·3
			1·48	14·2
			1·73	15·4
			1·56	15·3
			1·57	14·2
			1·51	14·0
			Av. 1·55	14·5

Three years later, Krogh (1937*a*, *b*) showed that fresh-water fishes and Amphibia are able to take up chloride from extremely dilute external media, and he later extended this work to other ions and to invertebrates (Krogh, 1938). His method was to keep the animals in a current of distilled water until their salt reserves were depleted and then to place them in measured volumes of dilute solutions whose final composition was determined by analysis at the end of the experiment. He was able to demonstrate active uptake of chloride in *Astacus*, in a variety of fresh-water molluscs and in the horse leech. Active uptake of chloride from dilute solutions has also been demonstrated by Koch (1938) for mosquito larvae, by Maluf (1939) for the earthworm, by Boné & Koch (1942) for caddis larvae and by Holm-Jensen (1948) for *Daphnia*. It is therefore of very widespread occurrence; but there are some fresh-water animals in which it has been looked for but not found, for example, in the eel and in the larva of the alder fly *Sialis* (Beadle & Shaw, 1950).

One of the invertebrates which Krogh studied in particular detail was *Eriocheir*, and he was able to show that there was active uptake of sodium, potassium, chloride, bromide, cyanate and thiocyanate; that nitrate diffused inwards rapidly under a concentration gradient, iodide slowly and sulphate not at all. He was also able to show that the mechanisms for uptake of anions and cations were independent, e.g. chloride, but not ammonium, taken up from NH_4Cl and replaced by bicarbonate; sodium, but not sulphate, taken up from Na_2SO_4 and replaced by ammonium. *Eriocheir* does not appear to show any discrimination between sodium and potassium or between chloride, bromide and thiocyanate when these are present in the same solution. *Astacus* (Schmidt-Nielsen, 1941) will absorb sodium but not potassium from solutions in which both are present, but does not distinguish between chloride, bromide and thiocyanate.

It so happens that for technical reasons the best evidence for active absorption of ions from the external medium comes from studies of fresh-water animals, and there is no doubt that among fresh-water animals these powers are well developed. Yet it would be wrong to suppose that they are wholly confined to fresh-water animals.

The most recent and most accurate analyses of the body fluids of marine invertebrates are those of Robertson (1939, 1949). Although the body fluids of some primitive marine animals resemble sea water very closely they are never identical with it owing to the Donnan effect which is set up by the proteins. Robertson used the method of comparing the ionic composition of the blood drawn from the animal with that of blood which had been dialysed against sea water. In this way the Donnan effect is eliminated from consideration, and it is possible to ascertain how far the differences between internal and external media are actively maintained. Some of Robertson's figures are reproduced in Table 2. From this table it can be seen that no active transport of ions is needed to maintain the composition of the sea-urchin's coelomic fluid. In the case of the lugworm *Arenicola* the only ion showing a significant difference in concentration is sulphate. But these are the exceptions. *Pecten*, the scallop, *Loligo*, the squid, and *Cancer*, the edible crab, are all typical marine invertebrates showing, like *Maia*, virtually no osmotic regulation; yet it appears likely that mechanisms of active transport are at work to maintain the generally higher concentration of potassium.

From this we may infer that the active transport of ions by the surface membranes, which is largely responsible for the hypertonic regulation of brackish- and fresh-water animals, is not of itself a novelty of adaptive evolution but is more probably the specialization of a mechanism which was already in existence in their marine ancestors.

Table 2. *Concentrations of various ions in body fluid as percentages of their concentrations in dialysed body fluid*

		Na	K	Ca	Mg	Cl	SO_4
Echinodermata	*Echinus*	100	102	101	100	100	101
Annelida	*Arenicola*	100	103	100	100	100	92
Mollusca	*Pecten*	100	130	102	97	100	96
	Loligo	95	219	102	102	103	29
Crustacea	*Cancer*	108	120	119	51	97	87
	Carcinus	109	117	108	34	103	60

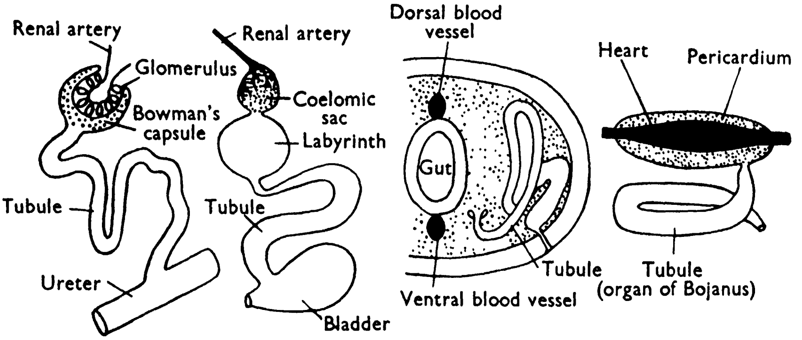
Carcinus figures from Webb (1940); the rest from Robertson (1939, 1949).

III. THE TRANSPORT OF IONS IN EXCRETORY SYSTEMS

The functional unit of the vertebrate kidney is the nephron which consists of a knot of blood vessels (glomerulus) projecting into a small coelomic vesicle (Bowman's capsule) from which a tubule leads to the exterior. The hydrostatic pressure of the blood in the glomerular vessels is sufficiently in excess of the colloid osmotic pressure of the blood to cause ultrafiltration, and the fluid in Bowman's capsule is identical with plasma except that it contains no protein. As this fluid passes down the tubule it is modified by the reabsorption of substances from it and the secretion of other substances into it. As is seen from Fig. 2*a*, the course of the fluid is: blood → coelom → tubule → exterior.

Now consider the excretory organs of invertebrates, some of which are shown diagrammatically in Fig. 2*b*, *c* and *d*. It is conceivably possible that the antennary glands of Crustacea are homologous with the nephridia of annelids, but it is not easy to refute the assertion that all these types of excretory organ have been independently evolved. Yet it appears that in all of them the course of the urine is the same, i.e. blood → coelom → tubule → exterior. If they have this much in common, is it possible that they have other features in common? Is it possible that, like the vertebrate nephron, they operate on the ultrafiltration-reabsorption basis?

The suggestion that the primary process of urine formation in invertebrates was a process of ultrafiltration was first seriously put forward by Picken (1936, 1937), whose main contribution to this thesis was to measure the colloid osmotic pressure of the blood in various crustaceans and molluscs and to show that it was always low in relation to the hydrostatic pressure. Ultrafiltration was thus a possibility. Paying particular attention to *Anodon*, Picken showed that the pericardial fluid was isotonic with the blood, whereas the urine collected at the excretory pore was hypotonic and therefore modified in composition during its passage through the tubule. He drew off the pericardial fluid and showed that it was continuously and fairly rapidly renewed. Further confirmation came from the work of Florkin & Duchâteau (1948), who found that the concentrations of calcium, chloride and phosphate in the blood and in the pericardial fluid were identical (Table 3).



(*a*) Vertebrate (*b*) Crayfish (*c*) Earthworm (*d*) Fresh-water mussel

Fig. 2. Diagrams of the excretory organs of a vertebrate and of *Astacus*, *Lumbricus* and *Anodon* to show the relation between blood system (black), coelom (stippled) and tubule.

For the others the evidence is less complete. In *Astacus*, Peters (1935) succeeded in withdrawing small samples from various parts of the antennary gland and determined the concentration of chloride (Table 4). His figures show that within the limits of accuracy the fluids in the coelomic sac and labyrinth are isotonic with the blood and that the urine becomes hypotonic during its passage through the tubule. I carried out similar investigations upon *Lumbricus*, measuring the freezing-point depression of blood, of coelomic fluid and of samples collected from different parts of the tubule. I was able to show that the coelomic fluid is isotonic with the blood and that the fluid passing down the tubule becomes hypotonic in the region known as the 'wide tube' (Ramsay, 1949).

Although these results are not in themselves sufficient to prove the truth of the ultrafiltration-reabsorption theory for invertebrate excretory organs they are at least compatible with it and can be said to raise it from the level of mere speculation to that of a reasonable working hypothesis. But we are still in the speculation stage in regard to the factors which have been at work in the independent evolution of this same physiological process in so many different animals.

Table 3. *Analysis of blood, pericardial fluid, Bojanus fluid and urine of* Anodon

	Blood		Pericardial fluid (%)	Bojanus fluid (%)	Urine (%)
	Absolute	%			
Chloride (mM./l.)	18·0	100	99	55	—
Calcium (mM./l.)	7·0	100	100	75	—
Inorganic phosphorus (mM./l.)	0·144	100	101	90	—
Δ° C.	0·06*	100*	100*	—	60*

'Bojanus fluid' is fluid withdrawn from the excretory organ through its internal opening into the pericardium, while 'urine' is fluid withdrawn from the excretory organ through its opening to the exterior. Figures marked * from Picken (1937), the rest from Florkin & Duchâteau (1948).

Table 4. *Chloride concentration (in mM./l.) in the blood and in fluids collected from different parts of the excretory organ of* Astacus

(From Peters (1935) as recalculated by Krogh (1939).)

Blood	Coelomic sac	Main labyrinth	End of labyrinth	Tubule	Bladder
196 ± 3	198 ± 2	209 ± 7	212 ± 7	90 ± 6	10·6 ± 0·6

In pursuing this line of thought we may next ask ourselves what happens in animals such as the echinoderms in which there are no recognizable excretory organs. These animals must take in water with their food and must produce metabolic water like other animals. How do they get rid of it? I am not aware that anyone has ever put this point to experimental test, but it would not surprise me to be told that this water simply escapes by seepage through the surface membranes of the body. I would be prepared to risk a guess that if fluid were injected into an echinoderm so as to distend its body and increase its internal hydrostatic pressure the result would be an outward seepage of salts and water, only proteins being retained—in fact, ultrafiltration through the thinner parts of the general body surface.

There is no serious disadvantage in this method of getting rid of water provided that the flow is normally very small—as it is likely to be in a marine animal whose blood is isotonic with sea water—and provided that the animal is not concerned to maintain the composition of its blood significantly different from that of sea water. But if the animal is maintaining some substance *X* in its blood in higher concentration than in the external medium and is actively transporting *X* against a concentration gradient, then it is wasteful simply to allow an ultrafiltrate to be swept away from the filtering surface by currents—for this reason, that less work is required to get X back again from the ultrafiltrate, in which it is *initially* at the same concentration as in the blood, than is required to get X from the external medium in which it is *always* at lower concentration than in the blood. This seems obvious, but it is only very recently that the point has been clearly put, by Potts.*

In the present context the interest of this conception lies in its evolutionary implications. We have seen the physiological parallels which can be drawn between the vertebrate nephron and the excretory organs of various invertebrates. Yet as far as we can tell they have all been evolved independently. I have suggested that ultrafiltration may be a widespread and primitive method of volume regulation; if this is true, then animals seem to have been at pains to restrict ultrafiltration to certain areas of the body and to arrange that the filtrate has to traverse some sort of tube before leaving the body. The point which Potts has made seems to me to provide the argument for selective advantage in this arrangement and enables us to understand why it has been evolved independently in different phyla of the animal kingdom.

If this is true it also implies that in marine animals having well-developed excretory organs the urine, although it may be isotonic with the blood, is probably not identical with the blood in composition. This is borne out by comparison of blood and urine in *Carcinus* (Webb, 1940) and *Cancer* (Robertson, 1939). In all these animals the urine is isotonic with the blood under all conditions, but as Table 5 shows there must be active transport of some ions in the excretory organ. As we saw in the case of the surface membranes, so now do we see in the excretory organs, that the active transport of ions, upon which depends the ability to penetrate fresh waters, is probably widespread among animals which are exclusively marine in habit.

Table 5. *Concentrations of various ions in urine as percentages of their concentrations in blood*

	Na	K	Ca	Mg	Cl	SO_4
Cancer (Robertson, 1939)	96	81	90	125	96	165
Carcinus (Webb, 1940)	95	78	94	390	98	224

It would appear therefore that the difference between *Maia* and *Carcinus* is not that *Carcinus* can actively transport salts while *Maia* cannot; it is likely that *Maia*, as well as *Carcinus*, has powers of active transport. The

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* I wish to thank Dr Potts for permission to quote his work which is as yet unpublished.

essential difference lies in the rate at which salts are transported *relative to the rate at which water diffuses passively in the same direction.* The degree of hypertonic regulation can be increased either by speeding up the active transport of salts or by reducing the permeability of the surface membranes to water, and we have perhaps paid too little attention to this second possibility.

There is some evidence which suggests that the surfaces of fresh-water animals are less permeable to water than the surfaces of their marine relatives. It is not easy to present this evidence in quantitative terms of permeability measurements because of the difficulties of measuring the surface area of an animal. But these difficulties are not insuperable, and it would be of great interest to know, for example, how far the success of *Eriocheir* as compared with *Carcinus* in penetrating fresh water is due to its powers of active transport and how far due to a decrease in the permeability of its surface to water. It would also be interesting to know if a decreased permeability to water is an active process in the sense that it demands a continuous supply of energy, as suggested by Beadle (1934) for the flatworm *Gunda.*

IV. OUTLOOK FOR THE FUTURE

Hitherto I have been concerned in tracing the growth of knowledge and ideas in what may be called the general field of osmotic regulation. In logical order, though not in chronological order, the problems were: first, to determine the general nature of the body fluids; secondly, to show that their composition was maintained by active transport; thirdly, to discover the sites of active transport in the body. There are, of course, a great many invertebrates, of which only a few have been studied, but as far as the major phyla are concerned it is fair to claim that sufficient ground has been covered to meet the first two points and it may be conceded that there is some progress to report in the identification of the sites of active transport. What is the next step to be? Are we to see the future merely as a process of filling in the details of a design whose main outlines are already clear?

I do not think so. On the contrary, it seems to me that the next few years will witness substantial changes in outlook.

Our present outlook is in fact still largely dominated by Claude Bernard and his 'fixité du milieu intérieur'. Primitive animals with no powers of regulation are condemned to live in the sea, those with some powers of regulation can work their way up estuaries and with the perfection of their mechanisms can graduate to fresh water. It is a good story and by and large it is true—but only by and large. On closer inspection the correlation between powers of regulation and ecological distribution is not so good.

The animals which penetrate farther up estuaries are by no means always those which can better maintain the constancy of the internal medium. *Carcinus* has greater powers of osmo-regulation than *Anodon*, yet *Anodon* can live in fresh water while *Carcinus* cannot. One need look no further than *Hydra* to find an animal which lives in fresh water and has no internal medium at all in Claude Bernard's sense of the term, and of these problems Dr Kitching is to speak later in this symposium.

The internal medium which Claude Bernard had in mind was of course the blood. But as has often been pointed out, the internal medium in which constancy is a prime requirement is the protoplasm of the cell. The responsibility for maintaining constant conditions in the protoplasm rests in the last resort upon the cell membrane. In the case of *Hydra* it rests solely upon the cell membranes throughout the body. But in the higher Metazoa living in fresh water, the task of the cell membrane can be made easier if the medium which bathes it is not fresh water but a saline solution whose composition is kept constant, and in so far as its task in this respect is made easier, so we may argue that the cell will be able to apply its resources more effectively to the main function for which it is specialized. What the animal does, in short, is to take a part of the load which would otherwise bear upon all the cells in its body and transfer it to those cells which separate the blood from the external medium.

The lack of close correlation between powers of regulation and ecological distribution need not therefore disturb us unduly. A marine animal may be able to get into fresh water either by evolving good powers of active transport in all the cells of its body or by evolving an internal medium which is kept constant by active transport on the part of a few cells in the body, those which separate the blood from the external medium. The first method may prove successful, but the second method lays the foundation of a more efficient physiological organization and has been adopted by Nature for all her greater evolutionary achievements. The second method, however, cannot be pursued to the complete exclusion of the other. Not all of the load can be transferred to the cells of the surface membranes, for, inasmuch as the cells of the body are not physico-chemically identical but vary from one tissue to another, the same internal medium cannot be in equilibrium with all of them and there is still some work to be done by their cell membranes.

And here, I think, is where our ideas need bringing up to date. We have been too ready to believe that once the internal medium is stabilized it is all over bar the shouting. We have concentrated too much upon the active transport mechanisms at the surface of the body and in the excretory organ. We have been inclined to think of the cells of the body as being able to relax, as it were, in a medium with which they are in equilibrium. And this in spite of the abundance of modern evidence which shows that the cells of the body are not in equilibrium with the fluid which bathes them, but are actively taking up some materials and are actively keeping others out.

One of the outstanding problems in this general field is presented by the inability, relatively speaking, of fresh-water animals to return to the sea. Although a great many animals have become successfully adapted to fresh water, not many have the power of passing freely from one medium to the other. Following Beadle & Cragg, complete adaptation to fresh water involves lowering of the osmotic pressure of the blood to a new general level of about half that of sea water. If a fresh-water animal is placed in sea water the osmotic pressure of its blood usually rises and the animal dies. The few animals which can survive transference from fresh water to sea water, such as the Salmonidae and the eel among vertebrates and the prawn *Palaemonetes* (Panikkar, 1941) among invertebrates, are capable of hypotonic regulation, that is, they are able to maintain the osmotic pressure of the blood below that of the medium when they are placed in sea water. To this the elasmobranch fishes form an interesting exception. The blood of marine elasmobranchs is isotonic with sea water by virtue of the retention of urea; the salt content of the blood is not widely different from that of fresh-water animals generally. This is interesting because it suggests that a high salt content rather than a high osmotic pressure *per se* is the decisive factor.

Beadle & Cragg investigated this problem on species of *Gammarus* living naturally in sea water, brackish water and fresh water and came to the conclusion that the ability of the animal to survive changes in the external medium was related to its ability to maintain differences in the concentrations of ions between tissues and blood as well as between blood and external medium. More recently, Camien, Sarlet, Duchâteau & Florkin (1951) and Duchâteau, Sarlet, Camien & Florkin (1952) have shown that there is a distinct difference between marine and fresh-water invertebrates in the amino-acid content of their muscles. The amino-acid content is higher in the marine species and makes a significant contribution to osmotic pressure. It may be that animals which have penetrated fresh water and have reduced the amino-acid content are unable to restore it when they are placed in sea water; osmotic withdrawal of water will then raise the concentration of salt in the muscles to levels which are higher than those characteristic of purely marine species and which the living cells may not be able to endure.

The converse problem is also met with; there is no doubt that the tissues of some animals are capable of working at high water content which in other animals would be unthinkable. *Anodon* has succeeded in entering fresh water not so much by its powers of active transport as by virtue of its ability to tolerate hydration of its tissues. The osmotic pressure of the blood of *Anodon* (and, so far as is known, of its tissues) is approximately that of 5% sea water—a quite exceptional figure—and the general wateriness of its tissues, to which Picken drew attention, is striking.

The moral of all this is that these are problems for the cell physiologist. In preparing this paper I was acutely aware that the problems and ideas which have guided research in this field have had an ecological flavour, whereas it is a common interest in processes of active transport at the cellular level, rather than in their ecological consequences, which brings this symposium audience together. But it seems to me that ecology cannot give the lead much longer and that for the future we must rather look to cell physiology for inspiration. The zoologist who seeks to interpret ecological distribution in physiological terms will have to concern himself more and more with the problems of cell physiology, and it is to be hoped that those whose interest is in the fundamental problems of all living matter will not overlook the avenues of approach to these problems which the invertebrates provide.

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VERTEBRATE PHYSIOLOGY FROM THE POINT OF VIEW OF ACTIVE TRANSPORT

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Active transport is presumably an essential feature of the vegetative activity of all cells, so that, in this respect, we cannot expect to observe striking differences according as we study organisms of increasing complexity, starting from the Protozoa, say, and finishing at the mammals; the individual cells of all these organisms will doubtless be shown to be capable of a high degree of active transport, and it may well be that certain highly differentiated cells of the more complex organism, e.g. the mammalian erythrocyte, will exhibit active transport to a less extent, and in a less varied form, than the Protozoa. The complex organism, however, because of its differentiation, exhibits certain structures in which active transport is not only necessary for their vegetative activity but also—and in a very high degree—in virtue of their specialized functions. Outstanding examples will spring to the mind: the stomach elaborating a solution of about 0·17 N-HCl; the kidney capable of selectively removing substances from the blood, the intestinal epithelium capable of the rapid absorption of selected substances from the lumen of the gut; the various glands producing characteristic secretions, and so on. Many of these specialized activities, involving active transport, I have had occasion to review recently (Davson, 1951), and a number of them, moreover, will be subjects of specialized and authoritative treatment in this symposium; consequently, in the present paper, I shall confine myself to a few general aspects of active transport taking place in specialized tissues.

Before discussing active transport—or secretory activity—it would be interesting and instructive to consider a form of transport in which simple physical forces appear to be adequate for the supply of energy involved in the process. The production of the glomerular fluid in the nephron, and of the interstitial fluid and lymph of the voluntary musculature, are examples. The glomerular fluid is, apparently, plasma minus the plasma proteins; the separation of this fluid requires energy to overcome the difference of osmotic pressure between it and its parent plasma, and this is provided by the pressure of the blood in the glomerular capillaries, i.e. by the mechanical work of the heart. The colloid osmotic pressure in the mammal is of the order of 30 mm. Hg, and there is little doubt that the glomerular capillary pressure is not only adequate to effect this separation of the plasma proteins, but also to provide the pressure-head necessary to maintain a continuous flow against the frictional resistance of the tubules. The separation implies, however, a membrane capable of holding back the proteins of plasma whilst permitting a ready flow of water and the smaller solute molecules of blood plasma; such a membrane is presumably given by the capillary walls, the intercellular spaces being sufficiently small to prevent—under normal conditions at any rate—the serum albumin and globulin molecules from passing through, but sufficiently large to allow inulin, gelatin and egg albumin to pass. The evidence in support of this intercellular route is largely presumptive; it is argued that it is unlikely that cellular membranes would show such a low level of discrimination as to permit the passage of all the non-colloidal constituents of the blood at the same rate, and, moreover, would permit substances of high molecular weight such as inulin to pass. In the case of the muscle capillaries an intercellular route for the flow of tissue fluid has been postulated on similar grounds; since, in this case, there is a very definite 'leakage' of proteins, we must assume either that the intercellular spaces are larger, or that the glomerular membrane of Bowman's capsule acts as a second, and more efficient, filter to ensure that only minimal amounts of protein find their way into the tubules. The evidence that the glomerular fluid is, indeed, nothing more than a filtrate from plasma, i.e. that no active transport mechanisms are involved in determining the relative concentrations of dissolved material in it and its parent fluid, is based on chemical analyses which, because of the very small amounts of fluid available, were probably not accurate to within less than ± 10%, although the large number of determinations carried out, and the absence of any trend indicating active transport mechanisms, make for a convincing body of evidence in favour of this simple origin of the glomerular fluid (Richards, 1938). The evidence with regard to the intercellular fluid of muscle is by no means so impressive, in fact I only know of one analysis of the relative compositions of plasma and this fluid, namely, that of the chloride distribution by Maurer (1938), so that the general physiologist bases his assertion that the capillary membrane exerts no active transport between blood and tissue fluid largely on the belief that the phenomena of fluid exchange between intercellular space and plasma are explicable on simple mechanical considerations (Danielli, 1940; Landis, 1934; Pappenheimer, J. R. & Soto-Rivera, 1948).

I raise this point not with the intention of shaking belief in the general proposition, but rather to show how inadequate such chemical evidence would be if it were desired to show that other tissue fluids, namely, the aqueous humour and cerebrospinal fluid, were likewise formed by simple ultrafiltration mechamisms. Thus an analysis of the main constituents of plasma and aqueous humour, e.g. Na and Cl, carried out within the limits of accuracy considered adequate for the study of the glomerular filtrate, would indicate an excellent agreement between theory and experiment, regarding the aqueous humour as a blood filtrate. Thus the ratio of the concentrations of sodium $(\mathrm{Na})_{\mathrm{Pl.}}/(\mathrm{Na})_{\mathrm{Aq.}}$ was 1·03 and that for chloride $(\mathrm{Cl})_{\mathrm{Pl.}}/(\mathrm{Cl})_{\mathrm{Aq.}}$ equal to 0·955, comparing with ratios, theoretically computed from the known base-binding power of the plasma proteins, of 1·04 and 0·96 respectively (Davson, 1939). Such a concordance was, indeed, so convincing that for some years I was ready to believe that the aqueous humour was, indeed, a plasma ultrafiltrate. However, the appearance of evidence against this view made me reopen the question; thus a deviation of 1 % from the equilibrium distribution of sodium, in this case, could be of profound significance; it could mean, for example, that the aqueous humour contained 1 % more NaCl and $\mathrm{NaHCO_3}$ than the blood plasma, a difference in concentration capable of maintaining a difference of osmotic pressure of some 60 mm. Hg, a by no means insignificant contribution to the forces driving fluid into the eye. The weak point in the work, however, was not the accuracy of the chemical analysis, which was easily high enough to permit the detection of a 1 % discrepancy, but the assessment of the theoretical Donnan distribution of Na and Cl for a dialysate of blood plasma, since the value of 1·04, given by Van Slyke (1926), postulates equality of activity coefficients in the two fluids.

Clearly the best way of investigating the matter would be to dialyse aqueous humour against plasma from the same animal, and see if there is any migration of Na and Cl from one fluid to the other. The results for the cat are shown in Table 1, the ratios for Na and Cl being determined before and after dialysis (Davson, Duke-Elder & Maurice, 1949). It will be seen that there is, indeed, a migration of both Na and Cl from the aqueous humour to the blood plasma; the true distribution ratios for a dialysate turned out to be 1·07 and 0·97 for Na and Cl and not 1·04 and 0·96 as computed by Van Slyke. This excess of salt in the aqueous humour is small, from the point of view of chemical analyses, and would have been quite undetectable by the methods used for the study of the glomerular fluid; nevertheless, it is large enough to influence the intra-ocular pressure and to rule out a simple filtration mechanism for the origin of the aqueous humour. I shall be returning to the problem of the aqueous humour later; for the moment I merely wish to emphasize the importance of, and difficulty in, determining the existence of active transport in certain systems. For many of the contributors to this symposium this has long ceased to be a problem, e.g. the transfer of salt by the frog's skin, the absorption of sugars from the intestine, and so on; and the problems have resolved themselves into determining the mechanism whereby the metabolic energy of the cell is made available for osmotic work. In the case of the aqueous humour and cerebrospinal fluid the problem has consisted primarily in demonstrating the existence of active transport mechanisms in the elaboration of these fluids.

Table 1. *Effect of dialysing aqueous humour against blood plasma on the distribution ratio of* Na *and* Cl

	$(\mathrm{Na})_{\mathrm{Pl}}/(\mathrm{Na})_{\mathrm{Aq.}}$	$(\mathrm{Cl})_{\mathrm{Pl}}/(\mathrm{Cl})_{\mathrm{Aq}}$
Before dialysis	1·042	0·945
After dialysis	1·068	0·971

A rather similar problem will doubtless arise with many of the more obvious forms of active transport; for example, we shall have to differentiate between the 'accidental' and the 'essential' in the composition of many secretions. Thus the obvious feature of the gastric secretion is the high concentration of hydrogen ions; the concentration of potassium is, however, about twice that of the plasma from which the secretion must ultimately be derived; the concentration of calcium is only about a tenth that in the plasma (Gudiksen, 1943). We must ask whether these differences are essential, in the sense that active transport mechanisms are operating on these ions, or whether they are the result of activity directed towards the hydrogen or chloride ion. A similar and more urgent problem, of course, arose with muscle, nerve and the erythrocyte. The evidence indicates that the active transport of sodium out of the muscle and nerve fibres is adequate to account for the accumulation of potassium, because the high internal concentration of non-permeating anions demands the replacement of the excreted sodium. It was originally suggested that the extrusion of sodium could explain the accumulation of potassium in the human erythrocyte (Dean, 1941; Maizels, 1949), but, as I argued elsewhere (Davson, 1951), this is to ignore the circumstance that the erythrocyte does not have the same high concentration of non-permeating anions; the extrusion of sodium would therefore only permit a limited accumulation of potassium, and to explain the observed accumulation an active transport of this ion must also be postulated (Harris & Maizels, 1952).

To come now to a more general aspect of secretory activity in specialized tissue, we may note that the outstanding feature of this activity—as contrasted with the vegetative activity in single cells—is the transport of material across an organized tissue; thus the accumulation of potassium by the erythrocyte or the extrusion of sodium by a muscle or nerve fibre, are processes that concern only the inside of the cell and a surrounding medium that may be considered homogeneous. Where transport across a tissue is concerned, we are dealing with an essentially asymmetrical system in which the medium surrounding the cells must be divided into two specific regions—the *donor* region, from which the actively tranported material is extracted, and the *acceptor* region, into which the actively transported material is driven. Between the two we have the cells capable of supplying the necessary metabolic energy. The asymmetry of this system must, in the last analysis, reside in the asymmetrical activities of the individual cells of the tissue, and it is worthy of note here that an important element in this asymmetry may be the organization of the cells in a definite layer; thus Chambers & Kempton (1933) showed that isolated cells of the chick mesonephros showed no evidence of accumulation of phenol red, whereas when organized in 'cysts' they did so. Viewing active transport, in these specialized tissues, as a transfer across an organized cellular structure, we must ask next whether the substances are indeed transported through the cells, and if so, whether they are accumulated to any extent within them. Again, we must pay attention to the role played by the spaces between the active cells; this role will, of course, be passive, but it is important to know to what extent the activities of the cells are favoured or prejudiced by the existence of regions in the tissue where diffusion may be as rapid as that observed in aqueous solution.

The transport of the secreted substance through the active cell is highly probable on *a priori* grounds—the metabolic activity of the cell depends on enzymes that are within it and, if chemical work is to be performed on a given molecule or ion, it seems reasonable to conclude that the molecule or ion must penetrate the cell to participate in the energy transformation. Nevertheless, it is worth pointing to an example of metabolic activity that seems to be located at the surface of the cell, namely, glycolysis of the erythrocytes of certain species; since glycolysis seems to be the basis for the energy available for the active transport of ions across the erythrocyte membrane, this point is not entirely irrelevant to the discussion. Wilbrandt (1938) showed that the permeability of the dog and rabbit erythrocytes to glucose was so small as to preclude the possibility of its metabolism within the cell. In the case of the rabbit erythrocyte this view seems to be borne out by studies of the effect of fluoride. This inhibitor of glycolysis actually causes a very rapid escape of potassium from the erythrocyte of this species; the effect seems to be dependent on the accumulation of intermediary products of metabolism, since it can be prevented by adding mustard gas which inhibits glycolysis in its initial stage, and it can also be prevented by removing from the system the necessary substrate constituents—glucose, phosphate, calcium, magnesium and potassium; the return of these constituents to the system causes the escape of potassium without any evidence of a delay due to the necessity for the magnesium, glucose, etc., to penetrate the cell. At what stage, if any, the glycolytic process becomes intracellular is not known, but even if the entire series of chemical reactions took place on the cell surface the energy liberated could presumably be made available for active transport if we accept Goldacre's mechanism for this process.

In certain cases the active transport of substances through the cells of the active tissue has been unequivocally proved; thus Chambers & Kempton, in the work previously alluded to, have demonstrated the presence of phenol red in the epithelial cells of mesonephros cysts during the process of accumulation; any real active transport, i.e. the transfer of phenol red against a gradient of electrochemical potential, was only definitely proved in the direction, cell to lumen, so that it may well be that in the donor region the passage into the cell is a matter of simple diffusion. Again, the secretion of HCl by the parietal cell of the stomach may be regarded essentially as a transfer of acid from the outside, donor, medium to the acceptor region in the canaliculi of the parietal cell; the intermediate accumulation of acid in the cytoplasm of the parietal cell must be ruled out by the observations of Bradford & Davies (1950). We may thus regard the secretory process, taking place across such specialized tissues as the tubular epithelium of the kidney or the gastric mucosa, as the penetration of certain substances into the active cells and their expulsion at another region; this latter process unequivocally represents active transport, but whether the former process, namely entry into the cells, involves any metabolic activity will depend on an analysis of the contents of the cell and the outside donor medium.

In this connexion we may note that many substances that are subjected to active transport are not substances that would be expected to cross the plasma membrane of a cell with any ease, e.g. glucose, or the sodium ion. It seems very likely to me, however, that a specialized form of permeability, not to be confused with active transport, will come into play in these cases. It was Danielli who first pointed out that glycerol penetrates into certain erythrocytes with a speed out of all proportion to what would be expected on the basis of measurements on comparable molecules, e.g. ethylene glycol, and he suggested the presence of active patches in the membrane in which the activation energy necessary for penetration was very low. About the same time I observed that, when cat erythrocytes were suspended in isotonic KCl, sodium leaked out with a permeability constant very much higher than that for the penetration of the smaller potassium ion; moreover, the permeability to sodium exhibited an optimal temperature in the region of 37° C., an optimal pH in the region of 7·4, and was markedly inhibited by narcotics, heavy metals, soaps, etc. The permeability to potassium, under identical conditions, was much more 'orthodox', being only mildly accelerated by narcotics and soaps, etc., and exhibiting a continuous increase with increasing temperature without any sign of an optimum. It was suggested (Davson & Reiner, 1942) that the permeability to sodium was mediated by an enzyme-like grouping that lowered the activation energy for penetration of the membrane; in other words, that the cell membrane had become specialized to permit the rapid migration of the sodium ion. A similar type of membrane specialization is probably at the basis of the extremely rapid exchanges of anions observed in the erythrocyte, a specialization that permits of the rapid acid-base exchanges in the blood when exposed to alveolar air for the short time available.

If I have understood Le Fevre's (1952) work correctly I would suggest that in the transport of hexoses across the erythrocyte membrane we have another example of this catalysed or specialized permeability. The essential feature of this permeability is that it is higher than what would be expected of an undifferentiated lipoid membrane, but it is a permeability that is observed with substances passing from a region of higher to one of lower electrochemical potential, i.e. active transport mechanisms need not be invoked. On the other hand, the specialization seems to take the form of an enzyme-like differentiation of the cell surface, so that the permeability is inhibited by narcotics, heavy metals, small shifts in pH, and so on. If this type of permeability is involved in the passive transfer into the secretory cells we may expect the active transport mechanism as a whole to be affected by narcotics, enzyme poisons, etc., even though the actual metabolic systems may not have been affected. This consideration must always be borne in mind when considering the action of enzyme poisons on active transport.

We have raised the question of the intercellular spaces in so far as secretory activity is concerned; we have asked whether their presence would be detrimental or otherwise to the process of active transport. The obvious answer is that they would be detrimental to any transfer of material against a gradient of electrochemical potential, in so far as they permitted back-diffusion from the acceptor to the donor region. We may consequently expect the intercellular spaces of a secretory epithelium to be small by comparison with the area of the cells. Thus, in the kidney tubule, glucose may be reabsorbed until there is no detectable concentration of this substance in the tubular fluid; this would suggest, either that back-diffusion is impossible—the intercellular spaces being too small to permit the molecule to penetrate—or, what seems more probable, that the rate of back-diffusion is too small to affect appreciably the concentration of glucose in the tubule in the face of the rapid process of active transport. Further evidence supporting the view that intercellular exchanges are not very significant has been provided by the work of Höber (Schmengler & Höber, 1933; Höber, 1933) on the frog kidney using the dual perfusion technique; according to these results, none of the sugars—glucose, galactose, fructose, etc.—pass from the blood to the tubular fluid when perfused by way of the renal portal vein, i.e. when presented only to the tubules. In those cases where passive diffusion from blood to tubular fluid appears to take place, e.g. with urea, thiourea, etc., it would seem that lipoid solubility is a prominent factor, indicating that this back diffusion is predominantly transcellular. Where secretory activity results in a marked difference in osmotic pressure between the parent fluid and the secreted fluid, the problem of back-diffusion—whether it be by way of extracellular spaces or across the cells of the secretory tissue—raises an interesting problem.

In general we observe active transport directed towards substances that penetrate cells slowly, e.g. ions, sugars, and amino-acids; highly lipoid-soluble substances are generally not transported actively. This is understandable, since the work done in maintaining a gradient of electrochemical potential depends directly on the permeability constant of the molecule concerned; to maintain, for instance, a concentration ratio of 20 between the inside and outside of the erythrocyte, the energy requirement would be of the order of a million calories per kg. per hour if the substance concerned were urea, far beyond the metabolic potentialities of the erythrocyte or of any other cell. From energetic consideration alone, therefore, we may expect secretory activity to be manifested towards slowly penetrating substances. The permeability of cells to water, is, in general, extremely high, much higher than the permeability of the erythrocyte to urea, for instance (Davson & Danielli, 1952; Collander, 1949), so that, where secretory activity results in a marked difference of osmotic pressure between donor and acceptor fluids, we must expect a modification in the cell membranes of the secreting cells in the direction of a reduced permeability to water, otherwise it would be impossible to maintain the difference of osmotic pressure. Thus the distal tubule of the mammalian kidney may establish a difference in concentration of the order of 1–2 M salt, equivalent to an osmotic pressure of the order of 50 atm.; again, the salivary secretion is strongly hypotonic, maintaining a difference of osmotic pressure of some 7 atm.; and in both instances the secreted fluid is separated from what is presumably a blood-isotonic fluid by only a single layer of cells. I know of no study in which the permeability to water of these cellular layers has been examined, and it would certainly be of interest to compare, say, the proximal and distal tubular epithelia from this aspect. The phenomenon of the maintenance of a large difference of osmotic pressure across a secreting tissue emphasizes once again, moreover, the limited area of the intercellular space in this tissue.

So far we have considered the intercellular space from a purely negative aspect; it is worth asking, however, whether active transport could conceivably take place through this space, i.e. essentially over the surface of the active cells, as opposed to through them. This, of course, is pure speculation, but I raise the matter for what seems to me to be a good reason, namely, the observation of secretory activity across multiple layers of cells, as in the plant root, the frog's skin, and the ciliary epithelium. The absorption of KNO_3 by the plant root is a process of active transport between the epidermal cells, which remove the salt from the soil or other nutrient medium, and the stele into which it is finally exuded as root-sap. Between this epithelium and the stele, however, there are successive layers of cells, and we have to consider whether the salt is actively transported by one cell-layer, excreted into the interstitial fluid in contact with the next layer, actively transported through the next layer, and so on, until it is finally exuded into the stele. This would appear to be a most inefficient process, involving separate acts of transport by each successive cell-layer. If a mode of extracellular active transport could be imagined, and at present I refrain from drawing any picture of a hypothetical mechanism, it might well provide a more efficient mechanism for the transport through successive cellular layers than one based on the more conventional view of active transport through cells.

The ciliary epithelium in the eye seems to present a similar problem, since it is made up of two layers of what appear to be secretory cells. Aqueous humour—essentially a solution containing the non-colloidal plasma constituents of which the main cation is sodium—appears to be secreted continuously from the cells of this tissue; the actual mechanism whereby the fluid is driven out of these cells is still a matter of speculation. In line with present thought on the active transport of sodium, we may say that the secretory cells actively extrude this ion from their inner surfaces into the acceptor region (the posterior chamber); as a result of this extrusion anions and water follow and the remaining constituents, e.g. potassium, sugar, urea, etc., may follow by simple diffusion from the secretory cells or through the intercellular spaces. If this is the essential basis, once again we must postulate a double process, involving secretory activity by the two layers in series. If the secretory cells contained normally a high internal concentration of sodium the work done in forming the aqueous humour would, of course, not be very high, and it would not be of much energetic significance if the process of formation were repeated by the second cellular layer.

One generally assumes that cells have a high internal concentration of potassium, in which case sodium must be excreted out of the cell against a high gradient of electrochemical potential and repeated excretion would have much greater energetic significance. It is worth remembering, however, that certain cells actually contain sodium as their predominant cation, e.g. the erythrocytes of the cat and dog. It would certainly be interesting to determine the potassium content of the cells of the ciliary epithelium. One hypothesis that I entertained for some time was that the aqueous humour was essentially an ultrafiltrate from the blood plasma, forced between the cells of the ciliary epithelium, and that these cells modified the filtrate by excreting sodium into it to produce finally a hypertonic fluid as found experimentally. If such were indeed the mechanism of formation of the fluid, the energy requirements would be low, the route of penetration of the various constituents being almost completely extracellular. On injecting various substances into the blood and measuring their rate of appearance in the aqueous humour we should not expect to find any marked differences in rates, since the theory postulates essentially a bulk flow through intercellular spaces. A detailed examination of this 'blood-aqueous barrier' revealed just the opposite, however, the rates of penetration of such substances as glucose, urea, sucrose, amino-acids, creatinine, etc., being so markedly different as to indicate that these molecules must pass a highly selective barrier—such as could be constituted by the ciliary epithelial cells—before penetrating. It seems very likely, therefore, that the aqueous humour is, indeed, elaborated within a cellular tissue and extruded from this as a characteristic secretion. It would appear from the studies of the secretory epithelia that they constitute, from the point of view of passive permeability, exceptionally tight barriers to diffusion of substances that normally pass through cells with great difficulty; in other words, that the intercellular spaces must be exceptionally small and by no means comparable with the extracellular spaces of the capillary endothelium.

The eye does, indeed, present an apparent exception which on further investigation seems to 'prove the rule'. Sucrose, raffinose and plasma proteins actually do penetrate the blood-aqueous barrier; because of the rapid drainage away of the aqueous humour, through non-selective channels, back into the blood, the concentration of, say, serum albumin, is only a small fraction (about 1 %) of the concentration in the plasma; nevertheless, this indicates a definite leak, presumably through intercellular spaces, of molecules of very high molecular weight. The evidence indicates, however, that the leak does not occur through the secretory ciliary epithelium but rather through the anterior surface of the iris over which the aqueous humour flows on its way out through Schlemm's canal. The rates of penetration of such high-molecular weight substances as have been examined, e.g. sucrose and raffinose, suggest that the substances pass through holes large by comparison with the size of the penetrating molecule (Davson & Matchett, 1953). It is probably in this manner that various antibodies and enzymes find their way into the aqueous humour. To speak teleologically, it is in this manner that the physiological disadvantages of a highly selective barrier, such as is constituted by a secretory epithelium, are overcome.

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THE CONCEPT AND DEFINITION OF ACTIVE TRANSPORT

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A glance at the titles of the contributions to this Symposium indicates that the problem of 'active transport' has become of considerable interest, not only for its own sake but also in relation to general problems of intermediary metabolism. New topics, such as nerve stimulation and recovery (Hodgkin, 1951), have been included, and certain observations (Lehninger, 1951) indicate that very similar phenomena of transport across membranes also occur in intracellular particles, especially mitochondria. In spite of the importance of the problem and the large amount of excellent experimental work which has been devoted to it recently, its detailed mechanism has been elucidated in scarcely a single case. The reasons for our meagre knowledge at the present moment probably lie mainly in the difficult nature of the problems and in the experimental inaccessibility of the systems which have been investigated. However, one must also consider the possibility that we have so far formulated our questions unfavourably owing to unsuitable basic concepts. This is suggested by the fact, among others, that at present active transport cannot be sufficiently characterized by numbers, and there is no clear definition of its concept which might serve as a base for a measure. Even if most workers in this field feel rather clearly what is meant by this concept, it is not primarily a phenomenon, pre-existing in nature, which can be recognized without being defined. There have been numerous analogous situations in the history of science. Carnot, in 1824, recognized rather clearly the physical meaning of the second law of thermodynamics, but he could not arrive at a definition of the concept of entropy. This was done 26 years later by Clausius, who could then give the general formulation of this law. I think that one should not regard questions of terminology as a mere formality. Hazy definitions and fundamentals are not only signs of the incompleteness of our knowledge, but also often the main obstacles to attempts to gain further theoretical and practical insight.

Fundamental definitions have varying functions in the study of a group of allied phenomena. In the early stages they facilitate the collection of the necessary empirical data by differentiating between superficially similar phenomena. In later stages they assist in the formulation of models and in the comparing of analogous groups of phenomena and thus in the understanding of the basal mechanisms. In the final stages they render possible the immediate recognition and classification of relevant phenomena in the study of new systems and the complete cataloguing of the whole group of phenomena. I think that as regards the problem of active transport we are in the second stage, and that therefore the replacement of more or less diffuse concepts by clearer definitions is of special importance. I would like, in connexion with these considerations, to quote a passage from Irving Langmuir: 'The progress of modern science depends largely upon (1) giving to words meanings as precise as possible; (2) definition of concepts in terms of operations; (3) development of models (mechanical or mathematical) which have properties analogous to those of the phenomena which we have observed' (Langmuir, 1929).

Early in the development of this field active transport has been contrasted with diffusion, which signifies the movement of a substance along a concentration gradient by reason of the thermal movement of the molecules. The quantitative aspects of the latter phenomenon had been worked out by Fick (1855), who formulated the so-called diffusion laws. Although the movement of a large number of substances across cell walls could be expressed satisfactorily by the diffusion equations, it soon became evident that these equations were not applicable to the movement of all substances in the living organism. The most obvious exceptions were found among the most important cell metabolites such as carbohydrates and amino acids, also cations and anions, water, and a number of other substances. For such exceptions the expression 'active transport' was coined, which was designed to convey the idea of the active participation of the cell in the movement of the substance. It includes the concept that the cell can use part of the energy derived from metabolism in regulating and influencing the rate and direction of transport. The significance of such an influence can be manifold: accumulation of certain substances within or without the cell in order to create optimum living conditions; the preservation of substances which are of importance to the cell or to the larger organism; or the elimination at increased rate of toxic substances or metabolic end-products.

If one tries to describe somewhat more closely such concepts as were gradually combined under the general heading of 'active transport', neglecting vitalistic considerations, one might say: by active transport is meant the transport of substances across one or more cell membranes which is influenced not only by the force responsible for passive diffusion, but also by other forces which are maintained and regulated by the metabolism of the cell.

The nature of the forces implied by such a description is unknown, as is their connexion with metabolism, and the question arises: when can one conclude that the transport of a substance is merely due to the force of diffusion? Generally it is difficult to reach a conclusion, partly because Fick's equations are not expressions of absolutely valid laws, but apply to ideal limiting conditions, and partly to our fundamental ignorance of conditions within the membrane. This forces us to make use of finite differences of concentrations instead of concentration gradients and to neglect structural properties of the membrane. For the estimation of the errors which are thus introduced experimental data are generally lacking.

Fick's well-known first diffusion equation is

$$s_i = -D_i A \frac{dc_i}{dx}, \tag{1}$$

where s_i is the amount of substance i which is transported per unit time across the cross-sectional area A normal to the direction of diffusion, D_i its diffusion coefficient and dc_i/dx its concentration gradient. This equation was originally set up by analogy to heat conduction rather than derived from first principles. Only considerably later did van Laar (1907) and Einstein (1908) work out derivations which under certain assumptions lead to (1). These derivations were based on the introduction of a force, the force of diffusion, which can be identified, in modern terminology, with the negative value of the chemical potential gradient ($-d\mu_i/dx$). The term 'force of diffusion' will be used in this sense here when applied to uncharged components. Assuming infinitely dilute solutions, expressed by $d\mu_i = RTd\ln c_i$, and a high resistance, one obtains an equation of the form (1); when not limiting the case to ideal conditions, the diffusion equation has to include an additional term with the gradient of the activity coefficient. In connexion with these derivations it is to be noted that the application of terms like force and resistance which are taken from analogous mechanical processes cannot be regarded as justified *a priori*, but needs the confirmation of empirical and statistical-thermodynamical methods. An extended diffusion theory, which also considers the effect of other forces, has been presented by Onsager (1931, 1945).

With regard to experimental evidence it is often taken as indicative of active transport, if one of the following factors exerts an effect different from that which it would be expected to exert on normal diffusion:

(1) *Concentration.* The rate is no longer a linear function of the difference in concentration on both sides of the membrane; especially at higher concentrations saturation phenomena may occur.

(2) *Competition by chemically similar substances.* This is analogous to the just-mentioned saturation phenomena.

(3) *Temperature.* The temperature coefficient may be unusually high, of the same order as that of enzymic reactions.

(4) *Slight structural modifications of the penetrating substance.* The rate can be completely different for structurally related substances, even those with similar molecular size or lipoid solubility, for instance, optical isomers.

(5) *Intensity of metabolism.* The penetration may often be dependent on simultaneous supply of oxygen.

(6) *Effect of enzyme activators and inhibitors.* In certain cases penetration can be completely inhibited by enzyme poisons.

Some of these phenomena show that the penetration of certain substances can be subject to regulatory mechanisms. Such mechanisms, however, need not be equivalent to the participation of additional forces or of the energy yielded by metabolism. One has to remember that although changes in the rate of penetration can be due to changes in the driving forces, they can equally well be due to changes in membrane resistance which depends on its structure. This structure in turn might depend largely on such factors as concentration, temperature, and occurrence of enzymic reactions remaining normal. Perhaps the biologist is often interested primarily in the existence of such control mechanisms and less in the question of whether they are due to changes in the resistance or of the driving forces. Since, however, there operate completely different mechanisms in the two cases, it seems desirable to differentiate between them.

It should also be mentioned—as Danielli (1943) has pointed out—that an 'abnormal' dependence of the rate of penetration on temperature and molecular size of the penetrating substance is to be expected if the passage of phase boundaries and microdiscontinuities are rate-determining steps. If at such phase boundaries only a limited number of free places is available to the diffusing substance, one might expect both saturation phenomena and competition. Dr Wilbrandt and I (unpublished) have calculated the case of a model with such an adsorption layer, and have found that in the case of the human erythrocyte the dependence on concentration of the penetration of glucose would not be inconsistent with such a structure. For other reasons, however, a carrier mechanism was suggested in this case. In addition, there is the often discussed possibility of a mosaic-like structure of the membrane with transport paths of varying chemical and structural specificity. Finally, it should be noted that even the dependence of penetration on enzymic reactions is no definite proof for its dependence on metabolism. It is possible, for instance, that a substance at the membrane undergoes an enzymic transformation independent of cell metabolism to an isomeric molecule, which might then be the one actually transported.

A method of testing for passive penetration, introduced by Ussing (1949, 1951), depends on new principles. It involves the application of two different isotopic forms of the diffusing substance, and has already yielded very valuable results, especially regarding the behaviour of inorganic ions. However, this method also has its limitations in the application to the present problem, as has also been pointed out by Ussing. I will not discuss the practical difficulties which arise when the method is applied to a substance, the participation of which, in metabolism cannot be neglected, but also for theoretical reasons it only gives an unequivocal answer to the present question if the substance under investigation does not undergo complex formation or any other interaction within the membrane either with itself or with other mobile membrane constituents. In such cases the diffusion currents of the two isotopes are not independent of each other. Thus in the case of the simple diffusion of benzoic acid through a layer of benzene we would find a deviation from the test equations for passive penetration, since in benzene benzoic acid occurs mainly associated as double molecules. An examination of the applicability of these methods to model membranes would appear to be of great interest.

The demonstration of transport from a lower to a higher potential (uphill transport) is a certain indication of the participation of forces other than of diffusion, and in my opinion, at the present time such a demonstration is the only certain criterion of active transport considering our ignorance of membrane structure and the available methods. For that reason I have, in an earlier discussion (Rosenberg, 1948), limited the definition of active transport to such cases. Even if such a definition appears as too narrow for many biological purposes it has several advantages, so that in every case a special treatment for uphill transport appears desirable. Thus one can draw conclusions as to a general mechanism by considering the numerous non-biological cases of transport against potential gradients. It is also possible to express a given uphill transport in terms of the amount of substance transported and the difference in potential. Finally, the demonstration of uphill transport is based solely on experimental evidence without requiring assumptions regarding membrane structure or mechanism.

A broader definition could be based on the above-mentioned description of active transport and formulated in the following manner: active transport is the movement of a substance which is influenced by other forces in addition to the chemical (or analogous) potential gradient of this substance. An advantage of this definition would be that it would roughly cover the usual concept and permit a theoretical treatment, whereas a disadvantage would be the difficulty of the experimental determination of whether a given transport is active if it is not an uphill transport.

I would further like to add that the thermodynamical and statistical treatments of irreversible phenomena during the last two decades have greatly improved our understanding of the problems of the transport of matter. Thus a number of systems which do not follow Fick's equations can be given a satisfactory and quantitative treatment by the above-mentioned theory of Onsager (1945), and also Brønsted's (1946) concepts have led to good results. These same theories, however, show us the limitations to which the applicability of all treatments up to now is subject. Thus, for instance, the integration of the relevant differential equations can only be carried out, if all conditions prevailing along the transport path are known. In Onsager's theory, values of potential difference and flow must be known for all quantities. Such theories are therefore of only limited value for the quantitative treatment of membrane systems of unknown structure with flows of unknown nature. A further limitation is that such theories are only valid for those systems which are not far removed from equilibrium. Transports which are induced by sudden fundamental structural changes of short duration can apparently not yet be treated in a satisfactory theoretical manner. Further, the possibility might be considered of whether in some case the very observation of a membrane transport is able to induce changes in the factors affecting the transport which cannot be neglected. Such a situation is known from atomic physics and is there expressed by the so-called uncertainty relations.

I will not consider these limitations in what follows and discuss a few somewhat simplified and schematic models in order to approach somewhat more closely the problem of the nature of the additional forces and their connexion with metabolism. Problems of the mechanism of the coupling with metabolic processes not only play a role in the case of active transport but also in the case of many analogous problems, e.g. muscular contraction or the formation of energy-rich phosphate bonds. In such cases one would often first ask the question: with which part of the total metabolism are these processes coupled? In the case of muscular work the required energy is evidently derived from the metabolism of carbohydrate. Such a conclusion does not, however, lead us to the mechanism of the coupling, for, as is known from the work of Lundsgaard (1930), the muscle can also work without the simultaneous utilization of carbohydrate as long as a reservoir of energy-rich phosphate bonds is available. We can illustrate the situation by means of a general scheme (Fig. 1). Let this scheme illustrate a metabolic reaction chain, for instance, the metabolism of glucose, which leads from the initial compounds G_1 via a number of more or less reversible reactions to the end-products G_n. Branch chains are linked to this main chain by coupling mechanisms, the nature of which is generally not known and which are represented by cog-wheels. For as soon as the nature of such mechanisms is known, they can be described by chemical equations or transports. That is due to the fact that from an energetic point of view a chemical substance can only either be transformed chemically or transported. The coupling between coenzyme oxidation and phosphorylation of ADP is thus at the present moment still to be represented by a cog-wheel, whereas the corresponding phosphorylation during the oxidation of phosphoglyceraldehyde can be largely represented by chemical equations (Warburg & Christian, 1939; Racker & Krimsky, 1952).

Assume that in such a branch chain there is a certain active transport, e.g. $A_5 \rightarrow A_6$. The conclusion that this transport is dependent on the metabolism of glucose is of course correct and can be important. However, it leads to no clue as to the nature of the link and the mechanism of the coupling. We can, however, consider this mechanism more closely by studying the process $A_3 \rightarrow A_4$ which yields directly the energy for the active transport. Although, of course, no specific information can be given concerning this process, one can make two general statements: (1) the process can also be described as a transport, and (2) the forces acting on it can be represented by the gradients of a limited number of thermodynamic potentials or homologous entities.

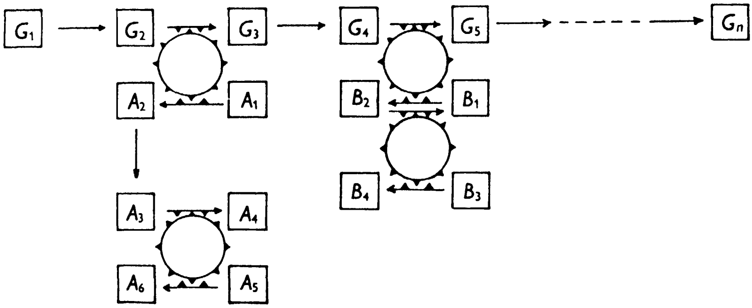

Fig. 1.

In this general form the above statements apply not only to such metabolic schemes but are also valid for non-biological coupled transport processes. For the purpose of illustration transports across cell membranes are, of course, among the least suited examples, since it is just there that we have no insight into detailed conditions. On the other hand, one can choose models from the numerous non-biological instances where the movement of a substance is not entirely due to the force of diffusion. Especially among the separation and isolation procedures in the laboratory there are several processes which involve the movement of a substance from a lower to a higher chemical potential. This occurs, for instance, in ultracentrifugation, electrolysis, distillation of mixture, electro-osmosis, thermo-osmosis and many others.

Before considering in more detail these or similar examples, let us examine a system which is regarded as normal with respect to the usual conditions of diffusion. Suppose a diffusion tube, placed horizontally, contains two substances i and k and is closed at both ends by membranes which are permeable to i and impermeable to k. The state in all parts of the tube is defined by conditions of constant temperature, constant pressure and by a stationary flow of i. Let the chemical potentials in two cross-section elements I and II separated by a distance of dx be $\mu_i + d\mu_i$ and μ_i. In addition to this system let us consider two phases I′ and II′ of the same composition as the two cross-section elements. During the reversible transfer of one mole of i from I′ to II′, assuming the absence of other compensatory processes, this system does work equivalent to $d\mu_i$ and loses an equal amount of energy. Let us assume an equal loss of work during the diffusion of one mole of i from I to II. The natural expression for the force causing such a movement is then the negative value of this loss of work divided by the distance dx. $K = -\frac{dA}{dx} = -\frac{d\mu_i}{dx} \equiv K_{\mu_i}$. On these relations are based the derivations of the diffusion equations.

Let us now consider the corresponding transport in the diffusion tube placed vertically. In this case the corresponding loss of work is no longer $d\mu_i$, for the transport of the substance involves a simultaneous transport of mass in a gravitational field. This portion of the energy change is represented by a term $M_i d\phi$, where M_i is the mass of one mole of i and ϕ the gravitational potential. The total decrease in energy during the corresponding reversible transport is therefore $d\mu_i + M_i d\phi$. The force acting on the transport, again represented by the negative value of the loss of work divided by the distance, is thus: $K = -\frac{dA}{dx} = -\frac{d\mu_i}{dx} - M_i\frac{d\phi}{dx} = K_{\mu_i} + K_{\phi_i}$. We thus have here an example of an additional force, K_{ϕ_i}, acting on the transport of a substance. This force is, of course, capable of causing chemical uphill transport, as happens, for example, in the ultracentrifuge.

In a similar manner chemical potential gradients can be used for lifting mass. An example of this is well known from text-books of plant physiology. It was originally designed by Askenasy and concerns the lifting of water in high trees (Hulett, 1903). The model consists of a vertical glass tube which is filled with water and is closed at the upper end with a porous gypsum plate permeable to water vapour. On evaporation of a certain amount of water through the gypsum plate an equivalent mass is raised from below to the top. We will not concern ourselves with the stability of the system nor with the correctness of its reproduction of the natural process, but rather with the energetic mechanism and the force which is responsible for the uphill transport of mass in this system. Since the water within the tube is in equilibrium, when no evaporation occurs, there must be—as we have just seen—a chemical potential gradient which in this case exactly compensates the effect of the gravitational potential gradient. We thus have in each cross-section: $K = -\frac{d\mu_i}{dx} - M_i\frac{d\phi_i}{dx} = 0$. The chemical potential of the water and therefore also its vapour pressure is thus lower at the top than at the bottom. The driving force for the lifting of the mass is thus the chemical potential gradient and the mechanism is the coupled movement of chemical matter and mass along the two potential gradients. The statement that here the energy is due to the evaporation is of course not incorrect, since the transport is due to the evaporation. It could, however, give the erroneous impression that the heat of evaporation or the work of evaporation are concerned in it. The value of the former, for 18 g. of water, is approximately 10,000 cal., that of the latter about 600 cal., whereas the work for the reversible raising of this amount of water through a tube, for example, 25 m. in length, would be 1 cal. This work equals the difference in the chemical potential at the top and at the bottom.

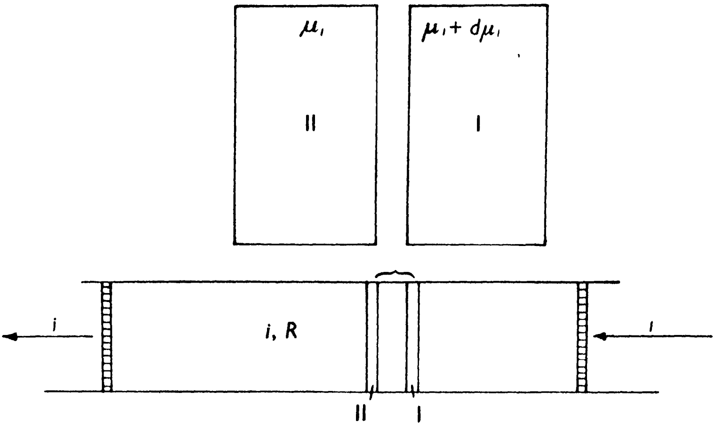

Fig. 2.

The result of these processes can be formulated as follows. When the movement of chemical matter is obligatorily bound with the movement of mass along a gradient of the gravitational potential, then there acts on this transport an extra force in addition to the chemical potential gradient.

Energetically therefore a complex of chemical matter and mass is conducted along the combined potential gradients.

We have based this result on the change in energy or the loss in work during the corresponding reversible transfer. Now, the energy equation in the form given originally by Gibbs has several terms, all of the form JdQ, where J represents an intensity with potential character and Q a quantity. This equation is an expression of the experience that differential energy changes can be specified quantitatively as thermal, spatial, mechanical, chemical, electrical, etc. In the same way, however, we are able to specify the forces involved in the transport of matter (or in other transports).

Let us again consider two phases I′ and II′ with the chemical potential $\mu_i + d\mu_i$ and μ_i and the pressures $p + dp$ and p. The reversible transport of one mole of i from I′ to II′ again results in the loss of work $d\mu_i$, but only on the assumption that no volume is transferred at the same time. If, however, we transfer together with i the volume v_i, then the total loss of work is given by $d\mu_i - v_i dp$. By applying this relation to the diffusion of a substance one can thus conclude that a pressure gradient can act as an additional force on transport and that such an action must be effective when under the given conditions the transport of matter and volume are dependent on each other. The total force on the transport is then

$$K = -\frac{dA}{dx} = -\frac{d\mu_i}{dx} + v_i \frac{dp}{dx} = K_{\mu i} + K_{pi},$$

where v_i is the volume which is being transported coupled to the transport of one mole of i.

These considerations show not only the formal similarity but also the difference between the action of the gradients of pressure and gravitational potential. When the latter is involved in a transport, there is always an additional force, $K_{\phi i}$, which is of constant value for a given transport. That is due to the fact that chemical substance cannot be transported without mass and that the ratio between quantity of matter (number of moles) and mass (number of grams) is always the same. Pressure gradients, on the other hand, do not act on the transport of matter if this latter is not coupled to volume transfer, and in such coupled transports the volume which is bound to the movement of one mole of i can be variable. The same conditions as in the action of pressure gradients obtain when temperature gradients are considered (entropy movement with a variable amount of entropy S_i per mole i) as well as the gradients of other chemical potentials $d\mu_r/dx$ (movement of substance r coupled to i with a variable amount n_{ri} per mole i). On the other hand, electrical charge transport is analogous to transport of mass if the substance in question does not during the transport participate in redox reactions and so alter its charge. It is therefore possible and also advisable for theoretical reasons to identify the force of diffusion of ions with the negative value of the electrochemical potential gradient: $K_{\bar{\mu}_i} \equiv -\frac{d\bar{\mu}_i}{dx} = -\frac{d\mu_i}{dx} - z_i\epsilon\frac{d\psi}{dx}$ ($z_i\epsilon$ = ionic charge, and ψ the electrical potential). In the same way one can, in systems in the field of gravity, define the force of diffusion as the negative gradient of a gravitational-chemical potential. Such a treatment is, as mentioned, not possible for the gradients of temperature, pressure and other chemical potentials. Since in these cases the corresponding quantities may vary during the coupled transport, the additional forces are also variable and depend on special conditions during transport. For the achievement of a chemical uphill transport, for instance, by a thermal force it is therefore especially favourable to choose a transport path along which the amount of entropy accompanying the substance is particularly large. The vapour phase is therefore favourable as transport medium for chemical uphill transport between two liquid phases.

Considerations of the properties of the transport path leads us directly to the answer of the question: Of what practical value are such seemingly abstract relations? The most important possibility is probably that the search for the additional forces and special mechanisms is given a certain direction. For this, examination of the conditions for the coupling of transports within the membrane is of special importance. Generally one would say conditions are especially favourable for coupling if an uncoupled transport of the isolated quantities concerned is difficult or impossible, that is, when the membrane is not permeable to the single quantities separately. For instance, an effective use of temperature gradients for chemical transports requires a relatively low thermal conductivity of the membrane. Effective chemical uphill transports are also dependent on low back-diffusion, i.e. on low permeability for the substance in question.

In connexion with this one can make the striking observation that in biological systems the substances which undergo definite uphill transports apparently all have a strongly hydrophilic character, whereas cell membranes under conditions of normal diffusion seem to be especially permeable to organophilic substances. Thus it would seem reasonable to search for organophilic transport complexes formed by the hydrophilic substances in question. This would direct attention especially on other chemical potential gradients as additional forces, for a hydrophilic substance can only become organophilic through chemical transformation. We deal here mainly with the question of models in order to approach more closely the problem of the driving forces and the mechanism of coupling. Not many models have been described in the literature illustrating the just-mentioned coupling, but the guaiacol models of Osterhout (1940) deserve interest in this context.

Let us examine more closely an example of these models. Two identical aqueous solutions I and II of potassium chloride and the potassium salt of guaiacol are separated by an organic, guaiacol-containing layer. The introduction of carbon dioxide into solution I then induces a transport of K^+ from II to I, which leads to the accumulation of potassium in I. In this uphill transport of potassium ions the guaiacol-potassium compound undoubtedly acts as the penetrating transport complex. The force which causes the transport of potassium ions therefore also contains a term with the electrochemical potential gradient of the guaiacol ion (G^-) and can, in complete analogy with the previously presented examples, be represented by: $K = K_{K^+} + K_{G-K^+} = -\frac{d\bar{\mu}_{K^+}}{dx} - n_{G-K^+}\frac{d\bar{\mu}_{G^-}}{dx}$. Here n_{G-K^+} gives the amount of G^- which is transported while coupled with 1 gram-equivalent of K^+. In a quantitative treatment of this model the expression would have to be modified because of a possible participation of undissociated guaiacol (GH) in the transport processes.

It is worth while to study this model a little further, for it illustrates two other problems which are of interest in this connexion. One is the dependence of the additional driving force, K_{G-K^+}, on a chemical reaction, the second is the chemical cause for the formation of the transport complex.

On introduction of carbon dioxide into solution I the following reaction takes place:

$$CO_2 + H_2O + G^- \rightarrow GH + HCO_3^-.$$

Since the guaiacol ion is consumed in this reaction there arises a concentration gradient or potential gradient of this ion. This acts as a driving force on the potassium ion, because the guaiacol ion cannot diffuse by itself through the organic phase, but only in combination with the potassium ion. We can express this connexion more clearly and generally by indexing the equation above:

$$CO_{2(I)} + H_2O_{(I)} + G^-_{(II)} \rightarrow GH_{(I)} + HCO^-_{3(I)}.$$

The connexion is thus based on the fact that the reacting substances, carbonic acid and the guaiacol ion, are separated by a layer or membrane and cannot come in contact with each other without carrying along the potassium ion.

The chemical problem is why the potassium compound is soluble in the organic medium, and this question is certainly not answered sufficiently by simple salt formation. General chemical experience shows that conditions for the existence of alkali ions as such in organic solvents are extremely unfavourable. Only such compounds can be expected to be organophilic in which the charge is effectively shielded. In the case of ions of heavy metals very many such organophilic compounds are known, especially the so-called chelate complexes. Thus the copper complexes of β-dicarbonyl compounds like acetyl acetone and the esters of acetoacetic acid and oxaloacetic acid are sparingly soluble in water and easily soluble in non-polar solvents. An uphill transport of copper ions by the mediation of these substances could thus easily be effected. Guaiacol also belongs to this class of chelate complex formers. The formation of complexes of this kind is due to formation of rings, usually of 5 or 6 members. Although the tendency of alkali ions for the formation of chelate complexes is much weaker, it nevertheless definitely exists as many findings have shown. While in the case of the ethylene diamine tetraacetic acid (Schwarzenbach & Ackermann, 1947) the sodium complex is more stable than the potassium complex, the reverse seems to be the case for β-dicarbonyl compounds (Sidgwick & Brewer, 1925). Several of the last-named complexes are easily soluble in toluene. Also the complexes with sodium and potassium of di-β-naphthol sulphide, containing an 8-membered ring, have a low solubility in water and are easily soluble in ether (Evans & Smiles, 1937). Several years ago we carried out a series of measurements of the electrical potentials in systems with artificial lipoid membranes to which were added such chelate complex formers (S. O. Nielsen & Th. Rosenberg, unpublished). We noted relatively strong potassium-binding effects of the esters of acetoacetic acid and oxaloacetic acid while sodium was bound less strongly. Dr Wilbrandt in Berne then studied the effect of diethyl oxaloacetate on the cation distribution in erythrocytes, but found none. One will thus have to look for more stable complexes for such effects. Also it is likely that the solvent properties of cell membranes are not characterized sufficiently by such descriptions as organophilic or lipoid.

It follows from the treatment which has been applied here that what we observe as active transport is the transport of only one part of an unknown transport complex, whereas the total transport of the whole complex is never active. In observing an uphill transport one can thus conclude that the rest of the transport complex in question is transported 'downhill' and that this furnishes, owing to coupling, the energy for the uphill transport. For this rest the term 'energetic carrier' was introduced in a previous paper (Rosenberg, 1948) because of its just-mentioned function. In order to avoid possible misunderstandings of this term, I would like to emphasize that there is no question of suggesting a special carrier mechanism of active transport, but merely of supplying a description which is essentially equivalent to that of the transport being under the influence of additional forces. The difference is mainly a specification of the forces which can act on transport of matter at all, and also of the conditions under which such an action becomes effective. On the other hand, the chemical organophilic transport complexes may be considered as a special application of these considerations of coupled transport.

Summarizing one can set up the following points:

(1) The concept of active transport should be clearly defined in the interest of the elucidation of the basic mechanisms.

(2) Such a definition cannot be based merely on deviations from the normal diffusion equations. First, one cannot presume that the diffusion behaviour is 'normal' during the penetration through structures like cell membranes, and secondly, we have frequently no possibility of differentiating between changes in the diffusion resistance and changes in the driving forces.

(3) A definition of active transport as transport under the influence of other forces in addition to the force diffusion (defined as the negative value of the chemical or electrochemical potential) is therefore only of limited practical applicability because of the difficulties in demonstrating such forces.

(4) A method introduced by Ussing, which is based on the use of isotopes, makes possible the demonstration of passive penetration if no specific interaction between the diffusing substance and mobile components, including the diffusing substance itself (association), occurs in the membrane.

(5) A chemical uphill transport, defined as transport from a lower to a higher chemical (electrochemical) potential, is proof for the action of additional forces, independent of the structure or of other processes in the membrane.

(6) Just like the force of diffusion, the additional forces may be represented by gradients of potentials or analogous thermodynamical entities. Gradients, as, for instance, the gravitational gradient, that of pressure, of temperature or of other chemical potentials, act as additional forces when the corresponding quantities mass, volume, entropy, or other chemical components move coupled with the substance in question.

(7) The general nature of the coupling during transport can be studied in well-known model systems. For the combined action of several chemical potential gradients the solubility of the transport complex and of its separate components is of decisive importance.

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SECRETION AND TRANSPORT OF WATER

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I. INTRODUCTION

There is now general agreement that ions are transported actively across cell membranes, but there is no such agreement about active transport of water. Water is certainly moved across cell membranes, but its movements might be secondary to those of ions. If ions are transported across membranes which are permeable to water, water must tend to follow because of differences in osmotic pressure set up by the alterations of ionic concentration. This is transport of water; it is even in a secondary sense active transport of water in so far as the primary transport of ions is active. But it is strictly passive so far as water is concerned, because the movement is one which, given the gradients of ionic concentration, would occur spontaneously. Rosenberg (1948) and Ussing (1949) have stressed that transport which can properly be called active differs in direction or in rate from what could be expected on the basis of gradients of chemical or electrochemical potential, and that, since it is not a spontaneous process, it can only continue so long as there is a supply of energy (e.g. from some metabolic source) to maintain it.

I want to consider the possibility that active transport of water in this stricter sense, even against osmotic gradients, occurs quite commonly in living systems—that cells can pump water, as such, with some sort of water-pump, and not merely secondarily by means of ion pumps. The evidence is not so complete or compelling as one could wish, but the possibility may have received less consideration than its deserves, and it leads to new ways of looking at old problems. That must be the excuse for following a rather speculative trail which future work may show to be false. It has been suggested that it is a beneficial intellectual exercise to practise believing impossible things, even if only before breakfast (Carroll, 1872).

II. FRESH-WATER PROTOZOA

Protozoa living in fresh water keep their osmotic pressure higher than that of their habitat; they accumulate ions and they require finite concentrations of metabolites. The concentrations within their cells have been measured in a variety of ways, e.g. by electrical conductivity (Gelfan, 1928), by vapour pressure (Picken, 1936) or by finding the lowest external concentration which will cause the organisms to shrink. This last method has been exploited particularly by Kitching (1934, 1936, 1938) in forms which possess contractile vacuoles. These vacuoles seem to excrete water which diffuses in from the environment because the osmotic pressure is higher inside the organisms. Metabolic poisons which stop the movements of the vacuoles cause the organisms to swell, and the external osmotic pressure which is required to prevent swelling when the vacuoles are not functioning can be determined.

Such experiments as these have suggested that the excesses of concentration on the inner sides of the cell membranes which form the body walls of Protozoa are of the order of 0·01–0·05 OSM/l. these correspond to osmotic pressures of the order of 170–850 mm. of mercury. Since it cannot be supposed that the cell membranes withstand differences of hydrostatic pressure of this order, the stability of these organisms cannot be explained simply by accumulation of ions and retention of metabolites within a semipermeable membrane. There seem to be three other explanations:

(1) The membranes are quite impermeable to water. This does not explain the osmotic behaviour of the organisms.

(2) The membranes are permeable to solutes as well as to water. This way round the difficulty is no more satisfactory. The contents of the organism would exert no osmotic pressure, but neither would they remain inside; they would escape and the organism would shrink. Active retention of any kind would presumably cause the membrane to behave as if semipermeable.

(3) Water is extruded actively as fast as it diffuses in under the influence of the osmotic gradient. This is the explanation which I prefer. When there is a contractile vacuole the extrusion of water can actually be seen. Kitching has shown how vacuolated organisms swell when the vacuoles are inhibited, and how the vacuoles cease to function if the ingress of water is checked by raising the external osmotic pressure. These organisms are not in osmotic equilibrium with their surroundings while they are alive, but they keep their volume constant and their contents hypertonic as a steady state by pumping out water. The energy which this requires must be provided by metabolism. Organisms which do not possess contractile vacuoles presumably pump water outwards across some part of the body surface by a mechanism which may be the same as that which transports water across the membrane of the contractile vacuole in a vacuolated organism. Kitching (1952) has pointed out that the emptying of the vacuole to the exterior could be explained mechanically by a very small excess of hydro-

static pressure, whereas a secretory process must probably be invoked to explain its filling. This secretion of water must be carried out across one layer of vacuolar or cell membrane, like the secretion of sodium which maintains a low concentration of sodium in mammalian cells bathed in sodium-rich extracellular fluids.

III. AQUATIC METAZOA

More complex fresh-water animals have body fluids which are considerably hypertonic, and marine teleosts keep their body fluids hypotonic to the environment. These fluids surround most of the tissue cells and provide them with a local environment with which they are usually supposed to be in osmotic equilibrium. Schlieper (1930) pointed out the important implication that the osmotic pressure of the body fluids needs to be carefully guarded, because it regulates the volume of the cells. We shall return later to the question how far cells and body fluids are truly in osmotic equilibrium. Meanwhile it is clear that, at the frontiers which separate the body fluids from the aquatic environment, there are layers of cells whose opposite poles are in contact with solutions which differ in osmotic pressure. It is not necessary to discuss the various devices which reduce the area of living tissue in the frontiers (shells and scales, for example) or which protect it, like the slime which seems to reduce the permeability to water of the body surface of eels (cf. Schlieper's reviews, 1930, 1935, and Krogh's book, 1939). Even if much of the surface is so shielded, there are osmoregulatory organs where the internal and external environments with their different osmotic pressures are separated by a layer of living cells. Here work is done to preserve the differences in osmotic pressure which are so important for the life of the animals.

IV. TERRESTRIAL ANIMALS

Frontiers of this kind which separate fluids differing in osmotic pressure also occur in terrestrial animals. Human saliva and sweat may have osmotic pressures half that of the fluid on the other side of the secreting epithelium, and greater osmotic gradients occur across the secreting epithelium which lines the convoluted tubules of mammalian kidneys. Human urine may be more dilute than sweat, and it may also be four times as concentrated as the plasma (Smith, 1951). The urine of some desert mammals may attain to higher degrees of concentration than this; the record seems to be held by a kangaroo rat studied by Schmidt-Nielsen, Schmidt-Nielsen & Brokaw (1948) with a urine of which the total concentration was nearly 6 OSM/l. The kidneys and the sweat glands therefore provide good examples of epithelia separating fluids with different osmotic pressures; moreover, the epithelia lining the renal tubules and the sweat glands are only a single cell in thickness, so that these cells must sustain huge osmotic gradients.

There may be a closer analogy than is at first apparent between Protozoa and the cells of osmoregulatory organs. The cytoplasm in the cells composing a living membrane which lies between two fluids with different osmotic pressures cannot be truly in osmotic equilibrium with both of them. Consequently an osmotic gradient must exist across a single layer of cell membrane at one pole of the cells at least. The production of anisotonic secretions has usually been regarded as a function of complete layers of cells, the secretory work being done in the cytoplasm. When the human kidney is producing maximal concentrated urine, the concentration of the fluid at the luminal pole of a cell lining the distal tubule may be 1·2 osM/l., compared with 0·3 osM/l. at the opposite pole, for there is evidence that the reabsorbed fluid under these conditions is at least as dilute as the plasma (Chambers, Melville, Hare & Hare, 1945). What then is the osmotic pressure of the cytoplasm? And where is the osmotic work carried out? Water must either be actively transported into the cells at the luminal poles, or else out of the cells at the opposite, basal, poles. In the former case, the cytoplasm must have a lower osmotic pressure than the body fluids in general for water to flow out spontaneously into the peritubular interstitial fluid in contact with the basal ends of the cells. This situation is not analogous to that in the Protozoa; it corresponds to a marine protozoon with hypotonic contents. In the latter case the cytoplasm must be kept hypertonic to the body fluids by active extrusion of water at the basal ends of the cells into the peritubular interstitial spaces. Reabsorption at the luminal pole could then be passive, for water would flow into the cells down an osmotic gradient. The urine could be concentrated up to a limit fixed by the maximal attainable intracellular osmotic pressure; but the limiting concentration would only be reached at low rates of urine flow because the rate of transport across the basal ends of the cells would set an upper limit to the rate at which water could be reabsorbed. Recent work by Ladd (1952) discussed by Homer Smith (1952) suggests that the final process by which the human kidneys elaborate a hypertonic urine may be a reabsorption of water unaccompanied by solute against an osmotic gradient (between urine and blood) at a rate of not more than 2–3 ml./min. Hence a process analogous to that which must operate to keep the contents of unicellular animals more concentrated than their surroundings could account for the known behaviour of mammalian kidneys, only it would have to operate at one pole of the cell, and not symmetrically all round its surface. There is an obvious histological difference between the basal and the luminal poles of the cells of the renal tubular epithelium. The basal ends show striations which are due to the parallel arrangement of mitochondria packed closely together at right angles to the basal surface of the cells. This would be consistent with a secretory process located at the end of the cell remote from the lumen; it is perhaps analogous with the grouping of mitochondria round contractile vacuoles, where a similar extrusion of water may be occurring.

To sum up, there is no doubt that differences in osmotic pressure exist across cell membranes in fresh-water Protozoa and in certain glands and excretory organs of higher animals. In the former active transport of water across a single thickness of cell membrane has to be postulated, and the same fundamental process could account for the other examples of secretion of water.

V. MECHANISM

Not much has been published to explain how such a process of active transport of water might work. Mechanisms like the diffusion pump which Franck & Mayer (1947) proposed require the cytoplasm to be divided into compartments by semipermeable partitions. They might move water from one end of a cell to the other end, and so pump it across a layer of epithelium, but it is hard to visualize them pumping water across one thickness of membrane either into a less concentrated environment or into a contractile vacuole. Thermo-osmosis provides a possible mechanism, which Dr Spanner is going to discuss. The alternate expansion and contraction of polypeptide chains combined with a cyclic alteration in their hydration might provide a pump within a membrane (Goldacre, 1952), and there are hints that the membrane may be a more complex organ than has been supposed. Sjöstrand (1953) has resolved some double membranes with the electron microscope in secreting cells and around mitochondria, which is interesting because Bartley & Davies (1952) suggested that mitochondrial membranes perform secretory work. There seems to be an association between double membranes and secretion, and they might contain the secret of the water pumps. But it is not yet known how water is pumped; an explanation may, however, be found if it is admitted that there is something to explain.

VI. OSMOTIC EQUILIBRIUM OF BODY CELLS IN HIGHER ANIMALS

Reasons have been advanced for believing that some of the cells in secreting organs cannot be in equilibrium with the body fluids which bathe them, and we must now return to the question whether the other cells of higher animals are truly in osmotic equilibrium with these fluids. It seems somehow natural and reasonable to regard cell membranes as semipermeable, and aqueous solutions separated by such membranes as in osmotic equilibrium. This hypothesis has been of great practical value in clinical medicine, for it has enabled alterations in the volumes of the various body fluids to be correlated with alterations in their composition, and it has satisfactorily accounted for most of the observed shifts of body water between the cells and the extracellular fluids. It explains, for example, the paradox that a deficiency of sodium produces a more severe form of dehydration than a pure deficiency of water (McCance, 1936; Marriott, 1947). Some account has been given elsewhere of how this simple and attractive view grew up (Robinson, 1953).

It was known that there was a greater total concentration of fixed base in the water in the cells than in the extracellular fluids, but this had been ascribed to a Donnan equilibrium, for the cells were presumed to contain considerable amounts of protein and other polyvalent and non-diffusible anions (cf. Newburgh's (1950) lucid account from the traditional standpoint). J. P. Peters (1935, 1944), who did perhaps more than any other investigator to preach the gospel of osmotic equality to the medical world, explained the apparent excess of osmotic material inside cells, and the fact that intracellular base sometimes varied without appropriate shifts of water, by postulating that a variable portion of the fixed base in cells is bound in some osmotically inactive form (Peters, 1937–8; Danowski, 1951). The manner of binding is unknown, and the amount bound can only be arrived at by assuming that the cells are in osmotic equilibrium, so that this assumption can only be preserved (as a dogma) by placing it beyond the possibility of experimental proof. The great attractiveness of this hypothesis can be seen from the fact that, if it is true, the osmotic pressure of the inaccessible intracellular fluids can be determined in living animals and men by analysing samples of extracellular fluids, of which blood plasma is typical and easy to obtain. But we have already seen that some of the cells in the body cannot have the same osmotic pressure as the extracellular fluids, and there is evidence which suggests similar inequalities in others.

Attempts to determine the osmotic pressure of cell fluids cryoscopically have been made from time to time since Sabbatani (1901) reported a greater depression of the freezing-point of water in the parenchymatous organs of dogs than in their blood. This sort of discrepancy has always been found, and seems to have been accepted at its face value by workers who have used the method, although the results might be affected by autolytic changes occurring in the organs *post mortem*. However, Sabbatani had found that the usual difference in osmotic pressure between the blood and the liver was abolished by poisoning with phosphorus, and Gömöri & Molnár (1932), who used the same method, found that differences in osmotic pressure between a number of organs and the blood of rabbits disappeared in the terminal stages of water intoxication. It seems that phosphorus poisoning and water intoxication had abolished either a normal hypertonicity of the cells, or the post-mortem autolytic changes.

VII. OSMOTIC PROPERTIES OF ISOLATED TISSUES

It has been known for some time that isolated tissues swell in solutions which have the same osmotic pressure as the body fluids which bathed the tissues during life. (See Robinson (1953) for some references to the earlier literature.) Opie (1949) found with pieces of the liver and kidney of rats that solutions of sodium chloride of about twice the osmotic pressure of the body fluids were needed to prevent this swelling, and suggested that the cytoplasm normally had a greater osmotic pressure than the extracellular fluids in the body. These results might also have been due to autolysis, if the membranes had remained impermeable to its products; but Opie (1950) found that the cells of the liver and kidneys swelled when the animals were poisoned with chloroform or potassium chromate, and that the behaviour of the isolated tissues then suggested that the poisoned cells, unlike normal ones, had been in osmotic equilibrium with their surroundings in the body. This was also true of the livers of rats poisoned with carbon tetrachloride, but the cells of those rats which did not die of the poisoning became hypertonic again when they recovered. These effects of poisons recall Sabbatani's (1901) old observation that phosphorus abolished the difference in freezing-point between the liver and the blood in the dog, and suggest that Opie's results also need not be dismissed as artefacts arising from autolysis.

VIII. OSMOTIC PROPERTIES OF SURVIVING TISSUE SLICES

The use of sodium chloride instead of a balanced saline medium might have contributed to the swelling of isolated tissues observed by Opie, but I have observed the same behaviour in thin slices from the livers (Robinson, 1952*a*) and kidneys (Robinson, 1950*a*) of rats in a medium which reproduced the ionic pattern of the extracellular fluids well enough to support respiration at a constant rate for several hours. Swelling was measured by the percentage of water in the tissue, for experiments with inulin had suggested that alterations in the amount of water in kidney slices reflected changes in the volume of the cells. The important point which emerged was that Opie's results were only confirmed when respiration was inhibited, for instance, by cyanide or by chilling to 0–4° C.; or when the metabolic reaction patterns were dislocated with 2, 4-dinitrophenol (Robinson, 1950*b*). Chilled slices swelled unless the medium had about twice the normal concentration of extracellular fluids, and the cells in slices poisoned with cyanide at 38·5° C. roughly doubled their volume in supposedly 'isotonic' solutions. They did not swell in these same solutions when respiring normally, and they swelled surprisingly little when respiring in hypotonic solutions.

An essentially similar relation of water balance to respiration had been reported by Aebi (1950*a*) for liver slices from the guinea-pig, and by Stern, Eggleston, Hems & Krebs (1949) for the liver, spleen, kidney, lung and brain of the same animal. The latter authors found that slices from all these tissues swelled under anaerobic conditions and concluded that some mechanism dependent upon the supply of energy was the dominant factor in regulating exchanges of fluid between the cells and the medium. Anoxia and cyanide might have damaged the cells irreparably, but the swelling which occurred when respiration was inhibited with cyanide turned out to be fully reversible when cyanide was distilled out of the medium and the oxygen uptake recovered (Robinson, 1950*a*). This suggested that Opie was right to believe that these cells were not in osmotic equilibrium while they were alive; but that their volume was regulated, and the osmotic pressure of their contents was kept above that of their surroundings, as a steady state, by some process which used energy derived from respiration to pump water outwards across the cell membrane. When the pumps stopped for lack of energy the cells had to swell. The action of 2,4-dinitrophenol suggested that energy was made available through the mediation of adenosinetriphosphate, and approximate calculations made for the kidney slices suggested that the amount of energy required to maintain the steady state was roughly proportional to the measured oxygen consumption, and that its absolute value was reasonable. Swelling in hypotonic surroundings would be anticipated if the pumps could not speed up to cope with the more rapid diffusion of water into the cells down a steeper osmotic gradient. The cells in slices respiring in hypotonic solutions did swell, but they swelled less than cells in osmotic equilibrium would have been expected to do, and also less than cells whose respiration was suppressed. Hence the volume of respiring cells reacted to changes in external concentration in the direction expected for cells in osmotic equilibrium, but to a smaller extent and for a different reason. I had made a few experiments upon liver slices before the work of Aebi and that of Krebs's team was published, with the idea that these would serve as controls and show the behaviour of kidney slices to be peculiar; but when the liver slices behaved in much the same way it began to look as though this sort of behaviour might be more general than I had supposed. This work has been published, and it remains to deal with a few points which merit further discussion.

IX. THE IMPORTANCE OF EXTRACELLULAR PROTEIN

Experiments upon tissue slices may fail in two main ways to reproduce conditions *in vivo*. First, Black (1953) has revived the suggestion of Trowell (1946) that the interstitial phase is a gel rather than a free fluid, but there is little information available upon this point. The fact that no extracellular fluid escapes from the cut surface of a dead tissue may mean only that water has been absorbed by cells which have been deprived of oxygen and have ceased to pump it out. Secondly, the media used in most manometric work contain no protein. It is not certain how much protein the interstitial fluids contain in the body: Drinker & Yoffey (1941) suggested that lymph might contain half as much as the plasma; but if the function of the lymphatics is to return to the blood stream protein which has escaped from the capillaries, lymph may contain more protein than interstitial fluid in general; how much more is uncertain. The cells of the renal tubules are probably surrounded by a fluid which contains very little protein, for the glomerular filtrate contains hardly any, the volume produced each hour is about 30 times that of the whole kidney, and all but a small percentage is reabsorbed. Hence a protein-free medium is probably physiological for experiments upon kidney slices, although the same may not be true of the liver, since this organ manufactures most of the protein of the plasma, and the hepatic lymph is especially rich in protein. In any case the colloid osmotic pressure of the extracellular fluids must be trivial compared with their crystalloid osmotic pressure.

Parry (1936) found that the proteins of dogs' serum did not prevent the swelling of excised portions of rats' muscle in 'isotonic' saline media. Aebi & Meyer (1951) found that liver slices from guinea-pigs only behaved as osmometers with respect to substances of high molecular weight when the cell membranes had lost their semipermeable behaviour with respect to ions (see later, p. 57). An earlier paper of Aebi (1950*b*) had shown that serum increased the oxygen consumption of liver slices by up to 200% by supplying metabolites, so that its action could not be ascribed simply to its colloidal osmotic effects. Suggestions that proteins prevent cell membranes from becoming leaky do not help; a cell might shrink if a leaky membrane allowed the contents to escape, but it should hardly swell. Finally, it is improbable that the use of protein-free media vitiated the experiments upon isolated tissue slices, because slices did not swell in protein-free media if their respiration was satisfactory, and the swelling which followed inhibition of respiration with cyanide was reversible in the absence of protein.

X. THE MAGNITUDE OF THE INTRACELLULAR OSMOTIC PRESSURE

The weakest point in this dynamic theory of the water exchanges of living mammalian cells is that it has still not been demonstrated directly that the intracellular fluids are hypertonic. Potts (1952) made some direct measurements by a micro-cryoscopic method and found that muscle cells of *Mytilis edulis* and eggs of *Psammechinus miliaris* were in osmotic equilibrium with their surroundings, but it remains to apply this method to cells which might not be expected to be in equilibrium. I estimated (Robinson, 1950*a*) that the cells of kidney slices might have an internal concentration

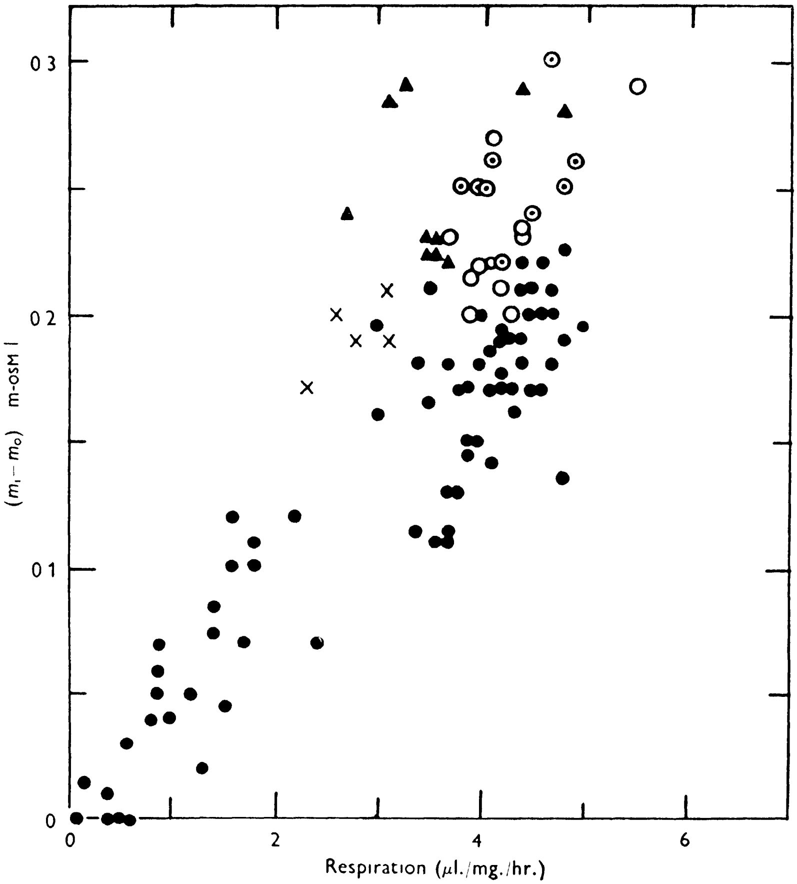

Fig. 1. Estimated intracellular hypertonicity and oxygen consumption of rat kidney slices respiring in media of different concentrations. Concentration of medium: ●, 0·30 osM/l. ('isotonic'), including experiments in presence of cyanide. ⊙, 0·19 osM/l. ▲, 0·06 osM/l. ○, 0·12 osM/l. ×, 0·03 osM/l.

of 0·52 osM/l. when respiring in a medium of 0·30 osM/l. The corresponding figures for slices respiring in media of other concentrations may be obtained, assuming the membranes to behave as semipermeable, by taking the internal concentration as $m = 0{\cdot}52/s_c$, where s_c is the relative volume of cell water determined from the observed percentage of total water in the slices ($s_c = 1{\cdot}00$ *in vivo*, when the external concentration $m_0 = 0{\cdot}30$). Fig. 1 shows $(m_i - m_0)$ plotted against the oxygen consumption of slices in 0·30 osM media with and without the addition of cyanide, and of slices in four more dilute media without cyanide, and reveals a clear relation between the estimated intracellular hypertonicity and the oxygen consumption of the tissue. Moreover, approximately the same concentration difference appeared to be maintained across the cell membrane in all these external solutions, whose concentrations ranged from 0·03 to 0·30 osM/l.

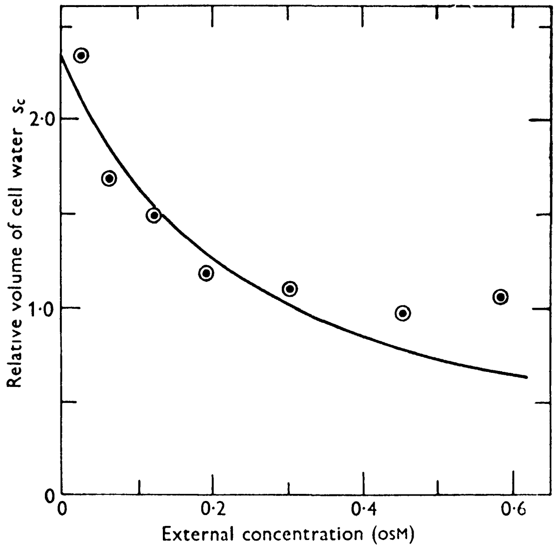

Fig. 2. Effect of concentration of external medium upon volume of cells in respiring tissue slices (expressed as 'Relative volume of cell water', s_c). ——, calculated from equation (2) ⊙, observed (Robinson, 1950*a*).

The amount of swelling to be expected if a constant difference in concentration was maintained across the cell membrane can be predicted and compared with that which was observed. The condition for a constant difference in concentration is

$$(0{\cdot}52/s_c) - m_0 = K. \tag{1}$$

But since *in vivo* $s_c = 1{\cdot}00$ when $m_0 = 0{\cdot}30$, $K = 0{\cdot}22$, and

$$s_c = \frac{0{\cdot}52}{(m_0 + 0{\cdot}22)}. \tag{2}$$

The smooth curve in Fig. 2 shows s_c calculated from (2); the points are the averages from experiments in different media. The cells did not shrink so much in hypertonic media as was predicted, but their behaviour in dilute solutions agreed well with equation (2). Moreover, (2) predicts a finite volume (corresponding to $s_c = 2{\cdot}36$) for slices in distilled water, whereas if the cells were in osmotic equilibrium and their membranes had remained semipermeable their volume should have become infinite. This point may not be merely of academic interest, for human urine can approach the concentration of distilled water quite closely. Hence the osmotic behaviour of the slices is consistent with a constant internal hypertonicity maintained by active transport of water.

XI. CONCENTRATION OF FIXED BASE IN CELLS

In an effort to gain more direct evidence of internal hypertonicity in respiring cells the total amount of fixed base (Na+K+Ca+Mg) was determined in kidney slices under various conditions (Robinson, 1952*b*). The concentration of fixed base was higher in the cell water than in the medium, and the difference was about the same in a 0·17 OSM as in a 0·30 OSM medium; but it could not account for a difference in total concentration greater than 0·07 OSM, compared with the value of 0·22 suggested for $(m_i - m_o)$ above. The larger estimate was based on osmotic behaviour and the smaller on determination of cations only, but it is generally supposed that electrolytes are responsible for most of the osmotic pressure exerted across cell membranes. An interesting point about this difference between the concentration of base inside and outside the cells was that it was abolished by chilling or by cyanide. The similarity between this and the effect of chilling and cyanide upon respiration, rather than the magnitude of the difference, suggested that it was due to an active process.

Some alternative interpretations must now be considered very briefly. The osmolar value of divalent cations is only half their chemical equivalence, but there is no reason to suppose that the cells contained enough divalent cations to account for the excess of total base without osmotic imbalance (McCance & Widdowson, 1946). Intracellular cations held by the electrostatic attraction of indiffusible anions should contribute to intracellular osmotic pressure. Conway (1945) suggested that most of these anions were substances of low molecular weight like creatine-phosphate, carnosine and adenosine triphosphate, which presumably also contribute to intracellular osmotic pressure. Hodgkin (1951) concluded that little if any potassium in the cells of excitable tissue is bound, and Klotz (1952) pointed out that no proteins so far examined bind sodium or potassium in complexes which should nullify their osmotic activity. Protein anions of large valency making small contributions to osmotic pressure might account for what has been called 'osmotically inactive base', though it is the accompanying anion that is relatively inactive. The amount of base that could be held in this way should depend upon the relation of the isoelectric point of the cell proteins to the intracellular pH, neither of which is known. It has been suggested that the contents of some cells are more acid than their surroundings (cf. Davson, 1951), and I did some very crude experiments which have confirmed this for the cells of kidney slices. These slices will accumulate phenolsulphone-phthalein (phenol red), a substance which is actively excreted by the kidney, for more of it may appear in the urine than in the glomerular filtrate formed at the same time (Smith, 1951). Slices from rat's kidneys incubated at 38·5° C. in the oxygenated medium used for manometric experiments also took up:

(1) tetrabromo-*m*-cresol-sulphonephthalein (= bromocresol green),
(2) dichlorophenol-sulphonephthalein (= chlorophenol red), and
(3) dibromothymol-sulphonephthalein (= bromothymol blue).

Table 1

Indicator	Bromocresol green	Chlorophenol red	Bromothymol blue
pH range	4·0–5·2	5·0–6·7	6·0–7·6
Colour of slice	Blue-green	Pink	Yellow
Slice + acid	Yellow	Yellow	No change
Slice + alkali	No change	Red	Blue-green

Table 1 shows the pH ranges of these indicators, the colours of slices which had taken them up from media buffered at pH 7·4, and the final colours produced after placing drops of N/10-HCl and N/10-NaOH upon stained slices. The colour changes produced by acid or alkali were delayed: nothing happened for about a minute, and then the colour suddenly changed, so that the staining of the slices was presumably due to dye within the cells, and the delay to the time taken for the reagent to penetrate the cell membranes. The cytoplasm in slices respiring in a medium of pH 7·4 thus appeared alkaline to bromocresol green, acid to bromothymol blue, and within the range of chlorophenol red, suggesting that the intracellular pH was around 5·5, but this must be regarded as an extremely rough estimate. A pH of 5·5 is not far removed from the isoelectric points of many proteins (Schmidt, 1945; West & Todd, 1951), although the isoelectric points of intracellular proteins other than those of muscle do not seem to have been determined. The amount of base held in cells by the electrostatic attraction of protein anions is therefore likely to be small, and it would not be easy to explain the swelling which occurs when respiration is inhibited by acid metabolites of low molecular weight lowering the intracellular pH and providing osmotically active partners for cations which were previously balanced by polyvalent protein anions of low osmotic activity. A large amount of swelling has to be explained, for the cells may double their volume when poisoned by cyanide. Moreover, only metabolic products to which the membrane is impermeable could account for the swelling, for although the increase in cell volume was complete in a few minutes, it was maintained for several hours. These considerations may make it easier to believe that the excess of base in respiring cells indicates an excess osmotic pressure of their cytoplasm.

XII. 'WATER PUMPS' VERSUS 'ION PUMPS'

If intracellular hypertonicity can be accepted, there seems little alternative to postulating active transport. Moreover, the cells swell when the transport system is deprived of its sources of energy. A transport system which opposes swelling must be directed outwards; and if it leads to a higher concentration inside the cell, it must be transporting water. The problem of how far the phenomena could be accounted for by, say, a sodium pump, and the swelling by water entering cells along with sodium when this pump is stopped, can be approached experimentally by working in sodium-free solutions. Kidney slices from rats respired satisfactorily, at least for an hour, when the sodium chloride in the usual media was replaced by choline chloride. Table 2 shows the percentages of water in slices in sodium-free media of different concentrations, compared with the corresponding values in ordinary media (Robinson, 1950*a*), both when the slices were incubated at 38·5° C. and when their metabolism was suppressed by chilling. When respiration was suppressed choline solutions prevented swelling slightly better than sodium-containing solutions, so that a small part of the swelling might have been due to the entry of water accompanying sodium. Columns 2 and 4 demonstrate the same inverse relation between swelling and respiration in sodium-free as in ordinary media. The differences in water content between column 4 and column 2 cannot be ascribed to the entry of water following sodium into the slices, because there was no sodium outside to enter. Slices also took up water in sodium-free solutions when their respiration at 38·5° C. was inhibited by cyanide. Hence respiration appeared to be opposing the entry of water, rather than that primarily of sodium.

In some experiments still in progress small concentrations of a mercurial diuretic (thiomerin) increased the amount of water in respiring kidney slices out of proportion to the effect upon oxygen consumption. Mercurial diuretics are generally supposed to act by stopping active reabsorption of sodium by the renal tubules, so that it appeared that this swelling might be the consequence of stopping a sodium pump. But further experiments showed that the effect of thiomerin was about as great in sodium-free solutions as in the ordinary media, which again points to a system transporting water independently of ions.

Table 2. *Percentages of water in adult rat kidney slices in ordinary and sodium-free solutions of different total concentration*

Concentration of medium	Chilled to 0–4° C.		Respiring at 38·5° C.	
osM/l. (1)	Na-free (2)	Na present (3)	Na-free (4)	Na present (5)
0·12	85·8	85·0 ± 0·8	81·9	81·9 ± 1·0
0·19	83·1	83·7 ± 0·8	78·5	78·5 ± 0·7
0·30	80·4	81·6 ± 1·3	77·5	77·5 ± 0·8
0·45	76·8	78·4 ± 1·5	76·6	75·4 ± 0·5
0·58	74·7	75·8 ± 1·8	76·5	76·5 ± 0·8

Ion pumps are presumably operating as well, and it is hoped that more work with mercurial diuretics may help to sort out their relations to the water pumps which also seem to exist. Aebi (1951, 1952*a*, *b*) made a detailed study of the influence of the conditions of incubation upon the properties of liver slices from guinea-pigs, and although he did not make use of metabolic inhibitors, his results resembled those I had observed with slices of the liver and kidney of rats. The main difference was that an inverse relation between the respiration and the water content of the slices was not always present, notably when calcium was omitted from the media, i.e. under relatively unphysiological conditions. Aebi's main conclusion was that conditions of incubation which were unfavourable for the retention of potassium within the cells were associated with a reduced consumption of oxygen, with swelling by uptake of water, and with more rapid disintegration of the cells shown by a faster loss of nitrogen from the slices. (The effects of lack of calcium upon the loss of nitrogen were remarkably similar to those published by Robinson (1949) on rat-kidney slices.) Aebi (1952*a*) suggested that the processes which controlled the water and the ionic contents of the slices were somehow linked, and perhaps possessed a common mechanism. Sodium entered the slices when potassium escaped, and this would be expected to follow the stopping of a sodium pump which normally kept the concentration of sodium in the cells low, and so kept that of potassium high, as has been proposed in the case of excitable tissues (Hodgkin, 1951). There is no reason on this hypothesis why the cells should swell if the sodium pump stops, for entry of sodium is balanced by loss of potassium (except for a slight swelling which might arise from the somewhat greater osmotic effectiveness of sodium ions compared with their equivalent of potassium; cf. Hill, 1950). Ion pumps might, however, influence swelling indirectly in a rather important way, if the functional semipermeability of the cell membranes were to depend upon the dynamic separation of sodium and potassium which they maintain. If sodium and potassium could diffuse freely across the membranes in both directions, external sodium chloride could exert no osmotic pressure, and its concentration would not be expected to control the volume of the cells osmotically. This may explain Aebi & Meyer's (1951) observation that the volume of the cells was controlled by colloidal osmotic pressure under conditions which prevented active osmoregulation and allowed intracellular potassium to be exchanged for sodium.

A sodium pump could perform two functions. It could maintain the characteristic difference in ionic pattern between intracellular and extracellular fluids, and thus also account for the apparent semipermeability of membranes which tracer studies have shown to be really permeable. It is doubtful how far a sodium pump could at the same time control the amount of water in the cell. Experiments in sodium-free media suggested that it could not account for the osmotic behaviour of the cells in surviving slices, and that there is in addition a water pump to provide independent control of the volume of the cells and of the tonicity of their contents. If these two separate pumps exist they are more likely to have a common source of energy than a common mechanism.

XIII. 'CLOUDY SWELLING'

If exchanges of water which influence the volume of the cells depend upon the metabolism of the cells as well as upon external osmotic pressure, it might be anticipated that shifts of water could occur in the body which were not the consequences of changes in the composition or the concentration of the extracellular fluids. A few examples of shifts of this kind have been produced experimentally, notably by Hamburger & Mathé (1951, 1952). They found that in poisoning with carbon monoxide or with sublethal doses of cyanide, and in experimental acidosis and histamine shock, water moved into the cells, with a reduction in the volume of distribution of thiocyanate, and an increase in plasma protein concentration and haematocrit, denoting a diminution in the volume of circulating blood. Shifts of this kind have really been known for a long time, although they have never been regarded in this light, for the phenomena of cloudy swelling are recognized in the parenchymatous organs by pathologists at a high proportion of post-mortem examinations. Cloudy swelling occurs especially in association with toxic conditions, fevers and anoxia. It is one of the mildest forms of cellular damage that can be recognized at autopsy, and in its earlier and less severe stages it is reversible (Bell, 1913; Moon, 1951). This suggests that there is a primary functional disturbance which precedes later modifications of structure. From the chemical standpoint the characteristic change is an increase in the amount of water in the affected tissues; the cells are swollen; yet they remain surrounded by body fluids whose osmotic pressure is not diminished. They resemble the cells of slices whose respiration is inhibited, and swelling of this kind is just what would be expected to occur if the water pumps ceased to function properly. Moreover, the results of Opie & Sabbatani (cf. p. 48) with poisons which produce cloudy swelling, suggested that these poisons abolished a normal hypertonicity of the cell contents. Cloudy swelling might be due to an increase in the amount of osmotically active material in cells in osmotic equilibrium; but it might also be due to the failure to maintain a normal steady state of disequilibrium. This might also explain the watery vacuolation of liver cells which Trowell (1946) found to be associated with anoxia, and to be reversible when the cause was removed.

A further characteristic of cloudy swelling is that the mitochondria within the cells are swollen (Türk, 1913; Anitschkow, 1914, 1923; Duthie, 1935). Mitochondria swell when the osmotic pressure of the cytoplasm is reduced by placing cells in hypotonic solutions. Opie (1948) suggested that cell inclusions of this kind might be regarded as intracellular osmometers. Zollinger (1948) showed that mitochondria swelled if they were released into isotonic saline solutions by rupturing the cell membrane, which suggests that the osmotic pressure of the cytoplasm is normally greater than that of these 'isotonic' solutions. This is also consistent with the experience of Hogeboom, Schneider & Pallade (1947, 1948) that hypertonic solutions had to be used in order to prepare isolated mitochondria from the liver and kidney of rats without the loss of their staining reactions. Hence the behaviour of mitochondria may indicate that the fluid within certain cells has a greater osmotic pressure than the surrounding interstitial fluid, although some recent work has made another interpretation possible. Bartley & Davies (1952) found considerable concentration ratios for some common ions between isolated mitochondria and the media in which they were studied; and Raaflaub (1952) discovered an inverse relation between the oxygen consumption and the swelling of isolated mitochondria in suspensions, which is reminiscent of the behaviour of cells in tissue slices. It is therefore possible that the behaviour of the cells might be the reflexion of the behaviour of the mitochondria.

There seems to be a choice here between hypertonic mitochondria bathed in a cytoplasm which is osmotically in equilibrium with the extracellular fluid, and mitochondria in osmotic equilibrium with a cytoplasm which is kept more concentrated than the extracellular fluid. Poisoning in the first case would stop secretion of water outwards across the mitochondrial membranes, and so the mitochondria would swell, and the cells would take in water to allow them to do so, but without increasing the volume of cytoplasm outside the mitochondria. In the second case poisoning would cause the cells to swell and come into osmotic equilibrium with their surroundings; at the same time the dilution of the cytoplasm would allow the mitochondria to swell as osmometers. The magnitude of the swelling which occurs when respiration of tissue slices is inhibited favours the second alternative; but in either case, whether an osmotic gradient is maintained across the mitochondrial membrane or across the cell membrane, there has to be a water pump somewhere.

XIV. CONCLUSION

The existence of osmotic gradients in Protozoa and larger aquatic animals as well as in secretory organs generally, indicates that certain cells can transport water actively across their membranes. The cryoscopic and osmotic properties of excised tissues, relations between the water balance of surviving tissue slices and their metabolism, the dependence of the concentration of intracellular base upon metabolism and the behaviour of mitochondria inside cells and outside them suggest that this process is not restricted to Protozoa and the cells of secretory organs, but that water is rather generally pumped across cell membranes. Cells in dynamic equilibrium with their extracellular fluids are less at the mercy of their surroundings than if they were in osmotic equilibrium in the classical, static, sense; their own metabolism allows them to take an active part in regulating the movement of water across their membranes. There is a certain fascination in the idea that the exchange of water between each cell and its surroundings may be an active one. Perhaps the only immediate value of this new way of looking at water metabolism is that it is provocative, and its irritant action may stimulate future research. Whether this is directed to showing how the water pumps work, or to filling the gaps in the orthodox formulation and destroying the source of irritation matters relatively little.

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ADDENDUM

Some additional comments are required in the light of further discussions and of publications seen since the foregoing went to press.

Hargitay & Kuhn (1951) showed how a small osmotic gradient maintained by some active process could be amplified to yield a larger difference in osmotic pressure between the fluids entering and leaving a system allowing counter-current diffusion, and Wirz, Hargitay & Kuhn (1951) demonstrated a gradual increase in osmotic pressure (measured cryoscopically) with increasing depth below the surface of the rabbit's kidney, as would be expected if the urine were concentrated by such a counter-current system. More recently Wirz (1953) found, in confirmation of this suggestion, that the blood in vessels in the renal papilla of the golden hamster had the same freezing-point as the urine. If this is the mechanism whereby the urine is concentrated, active transport is still required to provide a primary osmotic gradient for the counter-current system to amplify, but the osmotic gradient across the tubular epithelium need not be so large as was assumed in the discussion on p. 45.

An important paper by Conway & McCormack (1953) described cryoscopic measurements made upon a number of tissues with great care to avoid complications due to supercooling or autolysis. The results appeared quite conclusively to refute the hypothesis that the cytoplasm in liver, kidney or muscle is hypertonic to the surrounding extracellular fluids during life. On the other hand, Brodsky, Rehm & McIntosh (1953), who clearly set out to refute the same hypothesis, and who appear to have taken similar precautions, found that the osmotic activity of the intracellular fluids in the liver and kidney of dogs was always greater than that of the extracellular fluids by up to 50 %, in agreement with older work mentioned on p. 47.

The excess concentration of total base in respiring cells, mentioned on p. 53, was too small to account for postulated excesses of osmotic pressure, and might have been explained by the presence of polyvalent anions in the cells. More recently Aebi (1953) has reported concentrations of (Na + K) in slices of guinea-pig liver and kidney as much as 50–100 % greater than those in the media in which the slices were incubated. These values are probably too high to be ascribed to binding by polyvalent anions; indeed, they are greater than have been found in tissues removed from the body, but it may be that there is less disturbance of normal relations when a slice is quickly lifted out of its medium than when samples of tissue are removed from a dead or anaesthetized animal.

It is somewhat ironical that the pioneers who first suggested that some of the fixed base in cells is present in the form of osmotically inactive complexes thought of the amount bound in this way as small. Their analyses were made upon dead tissues, and there was only a small excess of base in the cells to account for. If recent chemical analyses of tissue slices and some recent cryoscopic measurements are both correct, it seems to follow that up to half the cations inside cells may be present in these inactive complexes, and it ought to be possible to isolate them and to elucidate their composition. But the last word can hardly be said until cryoscopic measurements as careful as those of Conway & McCormack have been made on the respiring tissue slices which appear to contain so much base on direct analysis.

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OSMOREGULATION AND IONIC REGULATION IN ANIMALS WITHOUT KIDNEYS

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I. OSMOREGULATION AND IONIC REGULATION IN COELENTERATES

In general the higher and more complicated animals excrete by means of kidneys, and to flush these there must be an uptake of water from the outside. This turn-over of water imposes on the organism a clearly defined problem in osmoregulation and ionic regulation—that of baling out water but of conserving valuable solutes, including ions, from the urine, and of making good from outside those which are lost. However, in small or very thin animals, provided there is no impermeable cuticle, there seems to be no reason why dissolved excretory nitrogenous products should not escape into the medium by diffusion. For instance, this is probably true of coelenterates and sponges. Even so, many coelenterates have formed an association with symbiotic algal cells which probably benefit by the supply of excretory solutes and help to remove them (Yonge, 1931). In any case there is no morphological evidence nor obvious physiological need for a continual exchange of water with the outside medium.

The coelenterates are a primitively marine group. They have only a very few fresh-water representatives, which include *Hydra* and *Limnocnida* (Hyman, 1940); *Cordylophora* lives in brackish as well as fresh water. Little is known about ionic regulation in the marine coelenterates. The jelly of *Aurelia* contains a slightly but definitely higher concentration of potassium than is present in the outside medium, and this is not due merely to a Donnan distribution, but must be the result of active transport by the cells which surround it (Robertson, 1949). There can be little doubt but that nervous action and muscular contraction depends on ion transport in coelenterates as much as in higher animals, so that in the coelenterates ionic regulation presumably came before osmoregulation. If excitation depends on the entry of sodium as well as the loss of potassium, one might expect that a fresh-water coelenterate would need to exercise some control over the ionic composition of its interstitial fluid and mesogloea as well as of the cytoplasm of its cells.

Nothing was known about osmoregulation in *Hydra* until the recent work of Dr Sylvia Lilly, not yet published. I am indebted to her for permission to refer to it on this occasion. She has shown that the tissues and cells of *Hydra* are rather highly permeable to water. Isolated tentacles of *H. viridis* were allowed to heal up and were then treated with solutions of sucrose. In 0·05 M-sucrose or stronger, the ectoderm, endoderm and lumen of the tentacles shrank, and it was also possible to demonstrate shrinkage of the ectoderm of intact *Hydra.*

Some information about the content of inorganic ions, and their rate of exchange, was obtained by Dr Lilly on *Pelmatohydra oligactis* by the use of radioactive isotopes. It is important to realize the nature of the information got by experiments of this kind. The organisms are transferred from the balanced medium with which they have been equilibrated to another which is chemically identical but in which a small proportion of the sodium consists of the radioactive isotope ^{24}Na. Some ^{24}Na enters in exchange for ^{23}Na, until at equilibrium the ratio of ^{24}Na ions to ^{23}Na ions is the same inside and outside. It is assumed that the organism cannot distinguish between the two kinds of sodium. Thus from a knowledge of the external concentration and radioactivity and from a measurement of the internal radioactivity at equilibrium and the total body water, the average internal concentration is calculated. If there is any internal unionized sodium which nevertheless for one reason or another is able to exchange with sodium ions, that unionized sodium will appear in the estimate obtained. On the other hand, if any sodium is isolated either morphologically or chemically from the ^{24}Na ions, it will not appear in the estimate. Thus the estimate obtained by radioactivity need not be the same as that which would be obtained by chemical analysis. For instance, according to Abelson & Duryee (1949) only 12% of the total sodium content of the frog's egg is readily exchangeable, the rest exchanging only very slowly; and it has been suggested that a part of the sodium content of human erythrocytes is not readily free to exchange under experimental conditions (Solomon, 1952). It seems likely that if there is any difference the estimate obtained from radioactivity is nearer to the ionized sodium than is that obtained by chemical analysis, although both kinds of information are important. However, the results obtained for radioactivity must be viewed critically, as the correction for decay of the isotope can lead to a considerable multiplication of the error.

To return to *Pelmatohydra*, this organism was found by Dr Lilly to come substantially into equilibrium in respect of ^{24}Na within about 12 hr. The average concentration of exchangeable sodium was found to be remarkably constant over a considerable range of external concentrations extending both above and below the average level. *Hydra* was also found to have a notable power of concentrating potassium, and to a small extent bromide. These results of course do not give any indication of localization within the organism; it is possible that sodium will be found predominantly in the mesogloea and potassium in the cells. In order to postulate a nerve and muscle physiology comparable with that of higher animals, it would be necessary to suppose this to be the case and to attribute to the outermost layer of cells in *Hydra* tissue the power to secrete sodium into the mesogloea or interstitial fluid, after the manner of frog's skin. The mesogloea would function as a primitive body fluid. Autoradiographic technique should decide this question. It is clear that the internal osmotic pressure exceeds the external, and that the osmotic uptake of water must be opposed actively or in some way compensated for by an outward secretion.

II. GENERAL COMMENTS ON PROTOZOA

There can be no doubt that ionic regulation plays an important part in the Protozoa. Many have conspicuous powers of excitation and contraction, contraction being localized in the ciliates in myonemes in the body wall and in some cases in a highly contractile stalk. Practically nothing is known of ionic concentration or distribution within Protozoa. For instance, it is not known whether the excitation of myonemes takes place at the plasma membrane or at an internal surface. The internal osmotic pressure of various fresh-water Protozoa is believed to exceed considerably that of the external medium (Kitching, 1951, and earlier papers), and the conductivity (Gelfan, 1928) suggests that this is largely due to ions. Work being carried out by Mr L. Carter, and still in an early stage, suggests that *Spirostomum* contains much more exchangeable potassium than sodium.

Although the various ions which are vital to life and activity no doubt contribute the greatest share of the osmotic pressure of the fresh-water Protozoa, some part must also be taken by proteins and other organic material in solution. Very little is known of the part possibly played by the body surface in transporting either ions or water, but it would be surprising if it did not transport ions. The contractile vacuole is probably concerned with baling out water which comes into the body by osmosis, and it seems likely that it possesses the power of retaining for the organism valuable ions. The physiology of contractile vacuoles has already been discussed at the 1951 symposium (Kitching, 1952), and I propose only to supplement this account by reference to recent work on osmoregulation in amoebae, and to the mechanism of vacuolar contraction.

III. OSMOREGULATION IN LABORATORY AMOEBAE

The contractile vacuoles of the various large laboratory amoebae have for a long time been difficult to fit into the osmoregulation theory of vacuolar activity. Adolph (1926) found that they continued their activity undiminished even when the organism was placed in a 0·05 M solution of NaCl or KCl. I suggested at the 1951 symposium that there might be a considerable time lag before a hypertonic solution reduced the rate of vacuolar output. Although in the long run an organism cannot continue to bale out water if none is coming in, it can continue to do so over a short period, and indeed the resulting shrinkage of the body may actually promote or determine the reduction in rate of vacuolar output (Kitching, 1951). Actually I had overlooked some interesting but obscurely published work by Belda (1942*a*, *b*, 1943) on the large multinucleate amoeba *Pelomyxa carolinensis*. Belda measured the body volume of *Pelomyxa* by drawing the organism into a capillary tube. He found that it shrank when placed in 0·1 and 0·2M solutions of non-electrolytes, but also shrank slowly and steadily, at a rate of about 0·33% of the body volume per hour, when kept without food in its own culture medium. After correction for the effects of starvation, it appears that the *Pelomyxa* approached volume equilibrium in the hypertonic solution in about 12 hr. In spite of the shrinkage of body volume, the contractile vacuole of *Pelomyxa* went on evacuating water, at a progressively slower rate, for some 80 min. after the organism has been placed in 0·1 M non-electrolyte; and during this time the body volume decreased by about 7–8%. There are differences of opinion about the internal osmotic pressure of amoebae. Mast & Fowler (1935) found that *Amoeba proteus* shrank when placed in 0·005 M non-electrolyte and regarded this as an upper limit to the internal osmotic pressure, but Belda (1943) has criticized this conclusion on the grounds that the shrinkage was probably due to starvation.

Belda accepted Gelfan's (1928) estimate of the conductivity of the cytoplasm of *A. proteus*, namely, 0·01 M-KCl, as the best available indication. However, Løvtrup & Pigon (1951), applying the correction for starvation to Belda's data for shrinkage in 0·1 M non-electrolyte made up in culture solution of very low concentration, concluded that the body volume decreased by exosmosis to about 80% and therefore that the internal osmotic pressure was that of an 80 mM non-electrolyte. If non-aqueous materials accounted for any considerable proportion of the body volume, this estimate would have to be lowered; but from Belda's data for shrinkage in 0·2 M solution it appears that the correction must be small. Løvtrup & Pigon also measured the vapour pressure of *Pelomyxa* raised in Pringsheim solution of osmotic pressure about that of 7 mM non-electrolyte. Batches of *Pelomyxa* were boiled or frozen in known quantities of distilled water. On four samples they obtained closely agreeing values averaging 107 mM non-electrolyte. They point out that this value may be too high owing to solution of additional material from crystals, and compromise on an average between their determination and that which they derive from Belda's work, thus obtaining an estimate for the osmotic pressure corresponding to 94 mM non-electrolyte—very much greater than that of the external medium.

For their study of the water relations of *Pelomyxa*, Løvtrup & Pigon took advantage of the possibility of using isotopic water for the estimation of the diffusion constant of water in the surface membrane. They also derived a relation between the diffusion constant of Fick and the permeability constant of Jacobs for water, so that from a knowledge of Fick's diffusion constant and of the difference in osmotic concentration on the two sides of the membrane it was possible to estimate the rate of osmotic entry of water into the organism. Under conditions of steady state this should equal the rate of vacuolar output.

The rate of penetration of isotopic water through the surface membrane of *Pelomyxa* was determined by Løvtrup & Pigon by means of the Cartesian diver. *Pelomyxa* previously equilibrated in a medium containing heavy water was transferred to the Cartesian diver in a drop of medium containing only ordinary water, or vice versa; and the changes in reduced weight were followed.

The diffusion constants for H_2O, D_2O and H_2O^{18} calculated from these experiments were not significantly different, and averaged $2{\cdot}6 \times 10^{-5}$ cm./sec., from which was calculated a permeability constant of $0{\cdot}011\ \mu^3/\mu^2/\text{atm.}/\text{min.}$ This is of the same order as that found in osmotic experiments for many other cells, but is at the low end of the range. From this permeability constant, the surface area of the *Pelomyxa*, and the difference of osmotic pressure, the rate of osmotic inflow of water was calculated to be about 2% of the body volume per hour, or perhaps slightly more. Belda found that the contractile vacuole evacuated 3·8% of the body volume per hour. Thus *Pelomyxa* differs from other Protozoa in which water relations have been studied only in that it is slow to react to a change in external osmotic pressure. This may be ascribed partly to the relatively low surface area, partly to its rather low permeability to water, and partly to a rather low sensitivity to the changes in body volume by which the contractile vacuole appears to be regulated.

IV. CONTROL OF RATE OF VACUOLAR OUTPUT

A study of contractile vacuoles may contribute knowledge of the control of the secretion of water which might have wide applications in other organisms or tissues, and therefore I have attempted to extend the suggestions made about this at the 1951 symposium. On that occasion I suggested that when the external osmotic pressure is changed, any lag in the response of the contractile vacuole to the new rate of entry of water from the medium into the organism will cause a change in body volume. This small change in body volume might well mediate the change in rate of vacuolar output. It was estimated that a decrease of 1½% in the body volume was associated with complete stoppage of the contractile vacuole in the suctorian *Discophrya piriformis*.

The rate of vacuolar output of the peritrich ciliate *Carchesium aselli* is also much affected by temperature (Kitching, 1948*a*), and there are reasons for supposing that temperature has an equivalent effect on the permeability of the body surface to water; so that the body volume, which depends on a balance of these two, remains unchanged (Kitching, 1948*b*). A study has now been made of the process of adjustment to a sudden change of temperature in the suctorian *Discophrya piriformis*. The response of the vacuole of this organism to a change of conditions is rather slow, so that a change of temperature which is relatively abrupt can be imposed.

The results of a rather drastic change of temperature are shown in Fig. 1. A rise in temperature from 6 to 20° C. caused an almost immediate stoppage of vacuolar activity, and several small kinks in the body surface gradually filled up. Then vacuolar activity was resumed at a rate of output considerably above the original. Thus the interpretation of the adjustment of the organism to the new temperature is rather hopelessly complicated by an effect on the secretory mechanism.

This effect may be exercised on the structural arrangement of proteins or lipoproteins supposedly concerned in secretion (Kitching, 1951). For instance, Goldacre (1952) has suggested that the folding of protein molecules offers a mechanism for active transport, and Marsland (1950) has shown that the viscosity of the cytoplasm of *Arbacia* eggs is increased by a rise of temperature. Without pressing this particular interpretation too far, we might suggest that high temperature opposes the structural change by which secretion is brought about, and so depresses or temporarily halts secretion, until accumulating reactants of the chemical processes involved and increasing hydration of the cytoplasm once more force the process forwards.

On return to a low temperature, in the experiment illustrated in Fig. 1, the rate of vacuolar output remained high for a short time, but then fell. The body surface became wrinkled, and it was clear that there was a decrease in volume. The shrinkage shown in Fig. 1 is exceptionally great. It conforms with the hypothesis already advanced concerning the control of vacuolar activity, but does not add anything to it. However, the experiments also provide interesting information about the frequency and ultimate diameter of the contractile vacuole, which has led me to a reconsideration of the mechanism of systole.

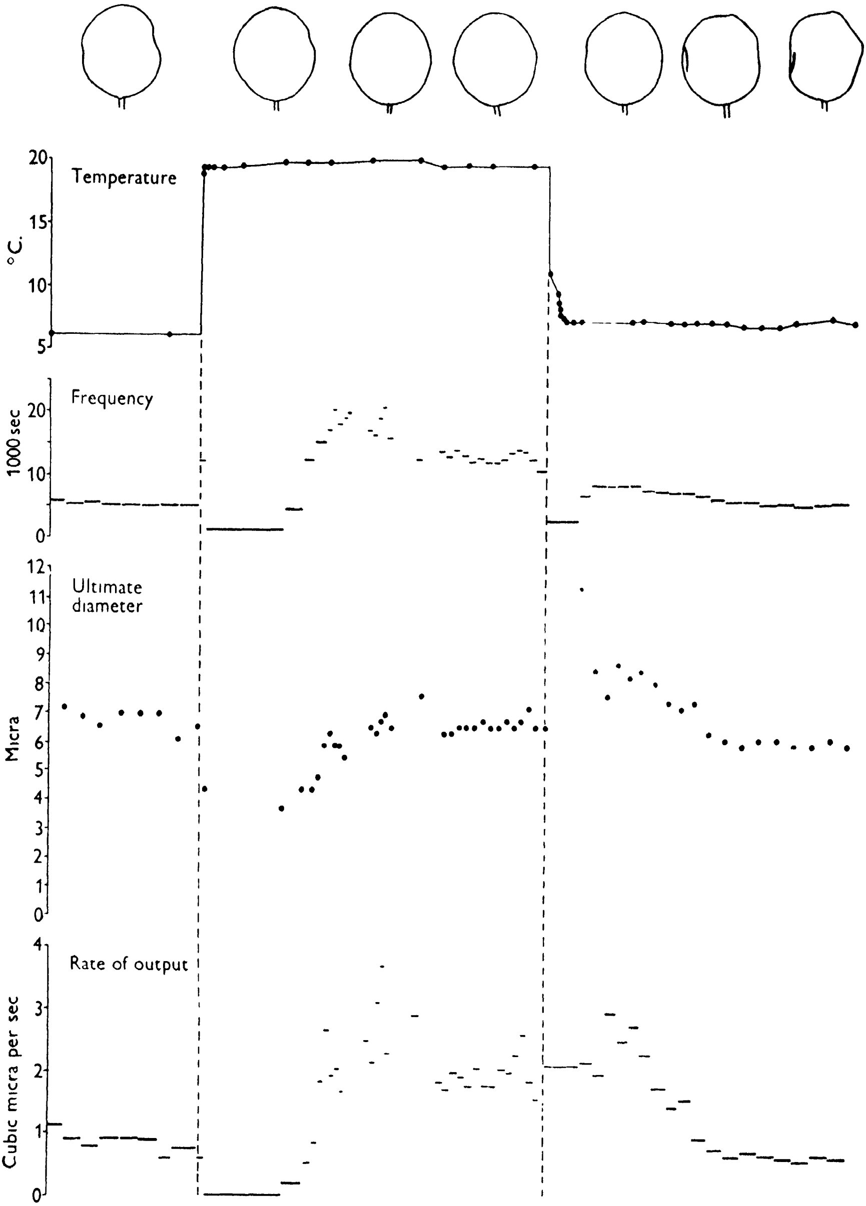

Fig. 1. Effects of a sudden change of temperature on the vacuolar rhythm of *Discophrya piriformis* Guilcher. This graph is plotted from data summarized by Kitching (1954*b*) elsewhere. The tentacles have been omitted from the drawings.

V. MECHANISM OF SYSTOLE

Since the 1951 symposium my views on the mechanism of systole have moved further in favour of a contractile vacuolar wall. The vacuolar wall is visible as a clear layer, about $\frac{1}{2}\mu$ thick in *Amoeba proteus* (Mast, 1938). If an *A. lacerata* is squashed the contractile vacuole may persist for some time suspended freely in water (Hopkins, 1946). The vacuolar wall of *Amoeba* is weakly birefringent, and the birefringence disappears at systole (Schmidt, 1939). These facts signify that the vacuolar wall is something a good deal thicker than the classical permeability barrier of the cell membrane, and it seems likely that the extra thickness is made up by a structural protein layer. A structural layer would clearly be needed to maintain the rather complicated shape of the permanent vacuolar apparatus in certain ciliates. Although any further discussion of this layer is bound to be highly speculative, it is natural to suggest a structure in which the long axis of the protein molecules lies in the plane of the wall, whether or not these molecules are contracted or partly contracted concertina-wise as in Mitchison's (1952) model. A structural protein layer might well possess elasticity, so that the vacuolar wall would become subject to increasing tension as the vacuole grew, and it might also possess the power of contraction (as suggested by Schmidt, 1939) under suitable chemical circumstances.

Body turgor is unnecessary for systole (Kitching, 1952). This is shown again by the effects of hydrostatic pressures of 2000–3000 lb./sq. in. (136–204 atm.) on the suctorian *Discophrya piriformis* (Fig. 2). The body surface has been thrown into creases (Kitching, 1954*a*), but the contractile vacuole undergoes systole quite normally and with increased frequency. Surface tension could easily provide the small pressure required if the vacuolar wall were liquid, but if there is in fact some rigidity of structure in the vacuolar wall it would probably be quite ineffective. MacLennan (1933) has described in Ophryoscolecidae a rounding-up of the contractile vacuole shortly before systole; then after some seconds the vacuole 'undergoes a sudden convulsion' and discharges. There is also a rounding-up some time before systole in various peritrich ciliates. MacLennan has reported that at the same time as the vacuole rounds up there is a solation of the neighbouring cytoplasm, as judged by Brownian movement, and he attributes the rounding-up to this solation. It seems very possible that the tension in the vacuolar wall which is necessary for this rounding-up is provided by the structural layer suggested above, and that a contraction of this is responsible for the 'sudden convulsion' of the vacuole at the beginning of systole. In any case it is necessary that the vacuolar wall should disintegrate as the vacuole contracts, and it is likely that in this process protein

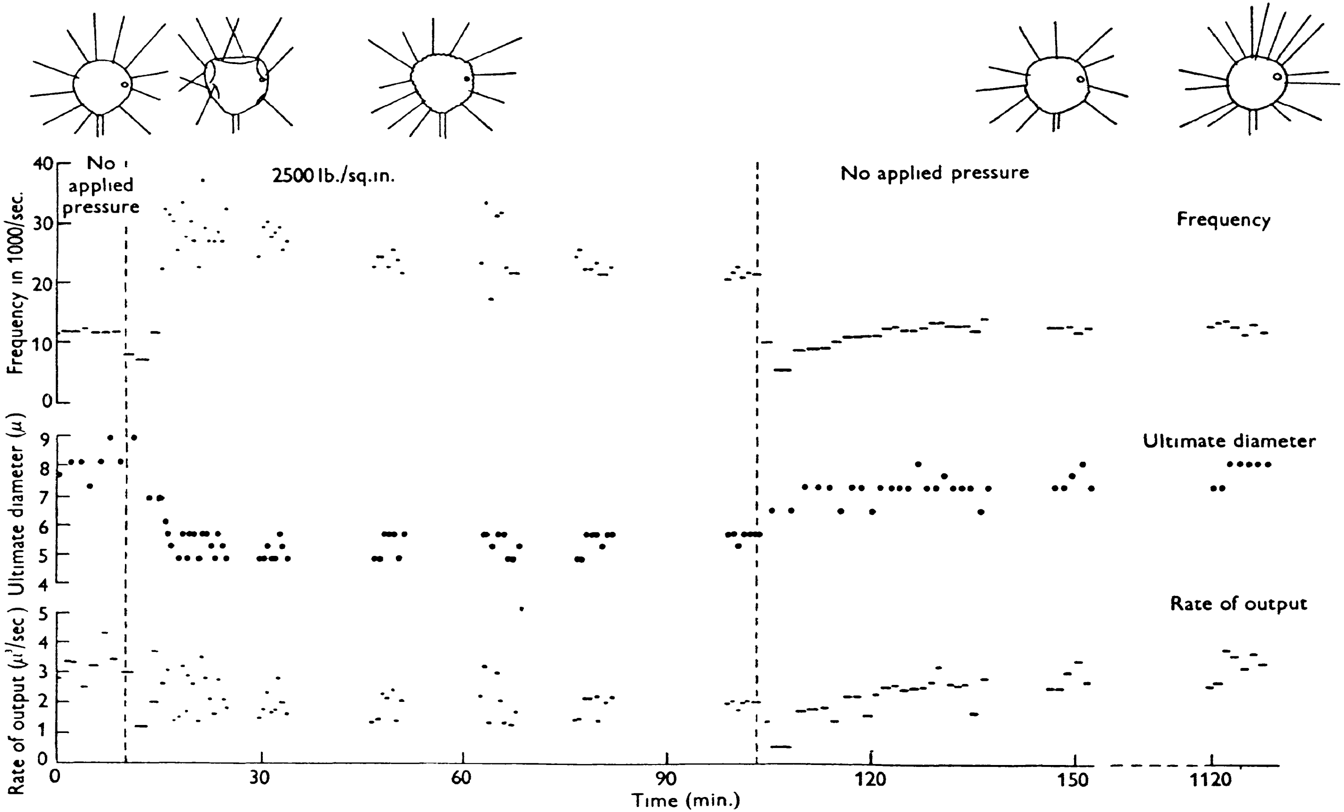

Fig. 2. Effects of a pressure of 2500 lb./sq. in. (170 atm.) on the activity of the contractile vacuole of *Discophrya piriformis*. (From Kitching, 1954*c*.)

molecules will fold and become globular and so pass into the surrounding hyaloplasm, much as postulated by Goldacre & Lorch (1950) for the tail of amoebae. During this process tension might be developed by the contraction of oriented proteins, or the dissolution of the structural layer might place the vacuolar boundary under the control of surface forces. As a speculation, it seems to me likely that the cell will use the contractility of proteins, which is their outstanding attribute, for the contraction which it must carry out. The remarkably complicated action of the contractile vacuoles of certain ciliates such as *Paramecium* (King, 1935) and *Haptophrya* (MacLennan, 1944) can more readily be explained if waves of contraction are admitted.

In *Amoeba* the contractile vacuole obviously must pass into the plasmagel before it discharges, and usually it contracts when in the tail. Here the surrounding plasmagel is itself contracting, and any cyclic changes of the chemical environment of the vacuole are likely to have reached a stage favouring contraction. In organisms with stationary contractile vacuoles, any such cyclic changes would have to take place at the vacuolar site. However, the rounding-up of the vacuole and the solation of the surrounding protoplasm described by MacLennan occur some 5–10 sec. before systole, and are regularly associated with it. It is reasonable to regard them as closely associated with some rhythmic process which sets the pace for the vacuolar cycle. Modification of the vacuolar cycle might be caused by modification of this rhythmic process. For instance, the characteristic overshoot in the depression of vacuole frequency, which occurs in *Discophrya piriformis* when the temperature is lowered suddenly (Fig. 1), might be ascribed to an effect of temperature on a series of chemical reactions which sets the vacuolar rhythm. Strong depression of one member of the series would slow the rhythm until the reaction products of preceding members accumulated and forced the process on again (Burton, 1939). Wells & Ledingham (1940) have made a model with water taps, a siphon and a kymograph to illustrate the application of this principle to rhythmic processes.

Let us consider now the characteristics of the vacuolar cycle at constant temperature. If the external osmotic pressure is lowered, the organism responds by an increase in rate of vacuolar output which is associated with an increase both in the frequency and in the ultimate diameter of the contractile vacuole. In some way both frequency and ultimate diameter are affected. Several mechanisms might be suggested: for instance, the small increase in body volume which supposedly mediates a change in the rate of secretion might also alter the condition of the pore plug and vacuolar wall in such a way as to make them more sensitive to the cyclic changes by which contraction is supposedly initiated, or the degree of extension of the vacuolar wall itself might influence the sensitivity of the mechanism to these cyclic changes. In either case, provided that the increase in frequency falls short of the increase in rate of secretion, the ultimate diameter would also be increased.

We may now return to Fig. 1 and consider the effect of a sharp rise in temperature on the vacuolar frequency. There is an immediate increase in vacuolar frequency. In fact, an overshoot would be expected, and is sometimes seen. However, the depression in secretion sets in and the frequency also falls, being linked with the rate of secretion. Only when the secretory activity is restored can the frequency attain the rate characteristic for the temperature.

The effects of high pressure on vacuolar frequency (Fig. 2) are interesting because they parallel those found many years ago on frog's heart by Edwards & Cattell (1928). At moderate pressure (2000–3000 lb./sq. in.) the vacuolar frequency is markedly increased, but at 5000 lb./sq. in. and upwards vacuolar secretion is strongly depressed, and with it the frequency also. Landau & Marsland (1952), in discussing the effects of hydrostatic pressure on cultures of frog heart, have suggested a general explanation in terms of a differential inhibition both of an enzyme-catalysed reaction and of the denaturation of that enzyme. Although this interpretation would give additional support to the idea of a rhythmic chemical process governing vacuolar contraction, not enough is known to justify its application at present.

VI. CONCLUSION

I will conclude with a return to the more general aspects of this paper. The fresh-water coelenterates appear to control their water content by an unknown but active mechanism and to take up various ions from the external medium after the manner of various other fresh-water animals (Krogh, 1939). Little is known about sponges, although some fresh-water sponges have contractile vacuoles (Jepps, 1947). The Protozoa control their water content in many cases by contractile vacuoles, and very probably will be found to take up ions through the body surface or part of it. It is not clear why Protozoa should have localized the secretion of water, but it is possible that in many cases the presence of a cuticle is concerned. This might well interfere with the outward secretion of water by the plasma membrane, and might also hinder the diffusion of dissolved excretory matter into the surrounding medium, thus making necessary an organelle with the function of a kidney. This view is proposed by MacLennan (1933) for the Ophryoscolecidae, into which the penetration of vital dyes was found to be much faster during feeding; they have a thick cuticle. The cuticle plays an important part in structural differentiation in the Protozoa, and it is suggested that its presence has rendered necessary the localization of secretion, and possibly excretion, at an internal plasma membrane, the vacuolar surface.

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THE ACTIVE TRANSPORT OF WATER UNDER TEMPERATURE GRADIENTS

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I. INTRODUCTION

In presenting this review on a particular aspect of active transport of water I want first of all to discuss the thermodynamics of transport processes in a general way to try to bring out more clearly the distinction between active and passive mechanisms, and to show just where the phenomenon discussed in this paper fits into the general picture. The procedure I want to adopt follows that of the relatively new Thermodynamics of Irreversible Processes which is destined, no doubt, to play an increasingly important part in biological theory (see, for instance, de Groot, 1951).

The meaning of 'active transport'

In a general way the meaning of 'active transport' is fairly clear. Whenever movement of matter occurs in a direction which we cannot explain as helping towards the attainment of equilibrium we recognize a case of active transport. Very often it is an extremely easy matter to decide whether we have such a case or not, but sometimes it is not at all simple, and our usual definitions land us in anomalies or fail us altogether. Consider one or two practical examples. Electro-osmosis can be either positive or negative, i.e. it can promote the movement of water in a direction either helping, or hindering, the normal *osmotic* flow. Now in both cases there is reason to believe that the mechanism is essentially similar; consequently if negative electro-osmosis is regarded as active (as it must be), so must positive electro-osmosis. In other words, an active movement can take place *down* a gradient of chemical potential or activity, as well as *up* one. Thus the very common definition of 'active transport' as being transport against a gradient of activity fails us—it expresses only half of the truth. Then consider the case to be discussed in this paper, where the temperature is not uniform. Here no special significance attaches to activity gradients at all; in fact, the difference in activity is only unambiguously known when the temperature is uniform. This makes the usual criterion of active movement really meaningless—a most unsatisfactory position. A knowledge of the real distinction between active and passive movements is obviously desirable, and I think it can be found by following the general approach introduced, I believe, by the Norwegian physicist Onsager in 1931.

Consider a very simple type of system (Fig. 1) divided into two equal halves by some sort of membrane, and containing water and a number of other substances. Imagine it at first to be in absolute equilibrium, so that the temperature, pressure and the concentrations of all its constituents are uniform everywhere. Further, let it be imagined to be entirely isolated, so that neither matter nor energy can enter or leave it.

Suppose we proceed now to disturb its equilibrium in a number of different ways. First, each one of the constituents can be redistributed between the two halves so that instead of being equal in amount on both sides it is now present in unequal amounts. Thus if glucose is a constituent there may be a mass m_g^{I} on one side of the membrane and a mass m_g^{II} on the other, and the extent to which we have upset the equilibrium can be specified by the difference $m_g^{\mathrm{II}} - m_g^{\mathrm{I}} = \Delta m_g$, which of course was originally zero. For each chemical substance present we shall have one degree of freedom in disturbing the equilibrium, and when we have finished, in imagination, displacing matter from one side to the other the state of our system can be specified by the variables Δm_1, Δm_2, Δm_3, ..., Δm_k, of which there will be one for each distinct sort of substance present. We can go further than this, however, and imagine that heat energy is made to flow from one side to the other, setting up a temperature difference ΔT between them. As a matter of fact, there will be many other ways still of disturbing the equilibrium (such as producing an electrical potential difference between the two sides, or promoting chemical changes), but for the purpose of illustration the matter need be taken no further.

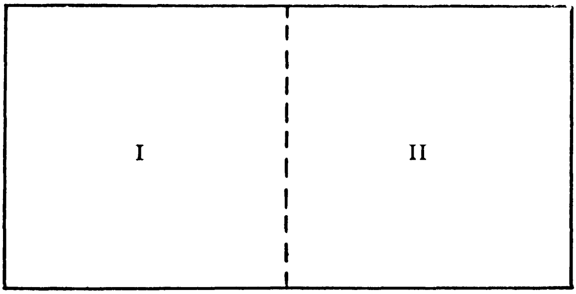

Fig. 1.

We now have a system not in equilibrium whose condition is completely specified by a number of variables Δm_1, Δm_2, ..., Δm_k and ΔT, and these, moreover, are all independent. In general, of course, our manipulations will have produced a pressure difference ΔP between the sides as well, but it will not be necessary to specify it, for it is already implied. In other words, it is not a further *independent* variable. We can if we so desire include it in the specification, but then not all our variables will be independent; one of them, say Δm_k, can be left out.

'Fluxes' and 'forces'

The foregoing example illustrates the point that when a system is not in equilibrium its momentary condition can be specified by means of a certain minimum number of variables $\alpha_1, \alpha_2, \ldots, \alpha_n$, all of which are independent. There is a certain latitude in choosing these, but their number is always the same, however they are chosen.

Consider now how the system returns to equilibrium. The one thing that can be said about it from the thermodynamic point of view is that its entropy continuously increases; it manifests a continuous tendency to increase its entropy, and when it can do so no more it comes to rest. It would seem reasonable therefore to regard the derivative $\partial S/\partial\alpha$ as measuring the tendency of α to change, i.e. as a measure of the 'force' changing it. Further, the time-derivative $d\alpha/dt$ can obviously be spoken of as the 'flux' of α. To make this clearer, however, let us refer again to the previous example. If $\alpha = \Delta m_g$ measures the extent to which the glucose distribution is 'out of balance', then $\partial S/\partial\alpha$ can be regarded as the force promoting transport of glucose and $d\alpha/dt$ as the rate of glucose transport.

Now the rate at which the entropy of the whole system changes can be written

$$\frac{dS}{dt} = \frac{\partial S}{\partial\alpha_1}\frac{d\alpha_1}{dt} + \frac{\partial S}{\partial\alpha_2}\frac{d\alpha_2}{dt} + \frac{\partial S}{\partial\alpha_3}\frac{d\alpha_3}{dt} + \ldots + \frac{\partial S}{\partial\alpha_n}\frac{d\alpha_n}{dt}, \tag{1}$$

by the basic property of partial differential coefficients, the partial derivative $\partial S/\partial\alpha_1$, for instance, being taken with all the other variables $\alpha_2, \alpha_3, \ldots, \alpha_n$ constant. The quantity dS/dt can thus be split up into the sum of a number of products of 'forces' such as $X_1 = \partial S/\partial\alpha_1$ and 'fluxes' such as $J_1 = d\alpha_1/dt$; furthermore, it is bound by the second law of thermodynamics to be positive. Calling $dS/dt = \sigma$, equation (1) can therefore be written in the more concise form

$$\sigma = X_1 J_1 + X_2 J_2 + X_3 J_3 + \ldots + X_n J_n, \tag{2}$$

a relation which is rather analogous to the electrical one

$$\text{watts} = \text{volts} \times \text{amperes}. \tag{3}$$

Equation (2) expresses how rapidly the system is increasing its entropy in terms of the instantaneous values of the forces and fluxes. However, these quantities are not independent; they can be related together in a way analogous to that in which the volts and amperes of equation (3) are related by Ohm's law:

$$\text{amperes} = \text{volts} \times \text{conductance coefficient}. \tag{4}$$

The 'Ohm's law' relations have, however, to be much more general, for it is a common observation that the movement of any one kind of matter promotes to *some* extent movement of all the other kinds present. Thus the flux J_1 will in general depend not only on the force X_1 but also on all the other forces X_2, X_3 and so on. Thus in general we must write

$$J_1 = L_{11}X_1 + L_{12}X_2 + L_{13}X_3 + \ldots + L_{1n}X_n, \tag{5}$$

and similarly for the other fluxes, the coefficients L_{11}, L_{12}, ... being 'conductance' coefficients whose magnitude will depend on the size and geometry of the system and on the nature of the mechanism of transport across the dividing membrane. By means of equations such as (5) (which are purely descriptive like Ohm's law), we can express the contribution of, say, the flux J_1 to the general process of increase of entropy. Calling this contribution σ_1 we have from (2) and (5)

$$\sigma_1 = X_1J_1 = L_{11}X_1^2 + L_{12}X_1X_2 + L_{13}X_1X_3 + \ldots + L_{1n}X_1X_n, \tag{6}$$

which is analogous to

$$\text{watts} = (\text{conductance of circuit}) \times \text{volts}^2. \tag{7}$$

Now the interesting thing about equation (6) is that the only term on the right which is *necessarily* positive is the first one; the others may clearly be either positive or negative. In other words, that part of the flux $J_1 = d\alpha_1/dt$ which can be attributed to the *conjugate* force $X_1 = \partial S/\partial\alpha_1$ necessarily contributes to an increase in the entropy of the system; that part which can be attributed to the *non-conjugate* forces, $\partial S/\partial\alpha_2$, $\partial S/\partial\alpha_3$ and so on, may contribute towards either an increase or a decrease in the entropy. This distinction would therefore seem to be just what is wanted to mark the division between passive and active movements; the passive component is due to the conjugate force, the active to the non-conjugate.

As a matter of fact this division is not entirely unambiguous, on account of the fact mentioned earlier that the variables $\alpha_1, \alpha_2, \alpha_3, \ldots, \alpha_n$ can be chosen in a variety of ways. However, the ambiguity can be overcome if the α's are so chosen that, in the simple example given, the fluxes $d\alpha/dt$ represent the rates of transfer of the individual chemical substances present together with the rate of flow of heat. Suppose this is done in a system containing water and one other chemical substance, say glucose. Then the *passive* movement of water on the present analysis proves to be associated with the force $\left\{v_w \Delta P + \left(\frac{\partial \mu_w}{\partial c_g}\right)_{T,P} \Delta c_g\right\}$, where v_w is the partial volume of water, ΔP the pressure difference, μ_w the chemical potential of water and c_g the glucose concentration per unit mass of mixture. Needless to say it represents no departure to attribute the nature of passive agents to the pressure and concentration gradients,† though if v_w should happen to be negative instead of positive the pressure gradient will operate in the reverse direction. The *active* movement of the water likewise proves to be due to the forces ΔT, the temperature differential, and $\left\{v_g \Delta P + \left(\frac{\partial \mu_g}{\partial c_g}\right)_{T,P} \Delta c_g\right\}$, where v_g is the partial volume of glucose and c_g is again the glucose concentration per unit mass. This emphasizes that any movement of water caused by a gradient in the chemical potential of another substance is necessarily an active movement; and that a pressure gradient has an active effect as well as a passive one, the active effect being dependent on $v_g \Delta P$. Further, and this will be the subject of the rest of this paper, any movement of water brought about by a temperature difference must be regarded as an active one, the criterion being, as mentioned earlier, whether it contributes *necessarily* or not to an increase in the entropy of the system.

II. THE IMPORTANCE OF TEMPERATURE

It is usually considered that as far as makes no difference living cells are isothermal systems. This is probably because the process of thermal conduction over minute distances is so extremely rapid that it is difficult to conceive of two points close together—say on either side of the plasma membrane—possessing temperatures measurably different. It seems to me that this view is in some respects inadequate, and elsewhere (Spanner, 1953) I have put forward the conception that where metabolism is active any given point in the cell possesses not *one* temperature, but many; in fact, one to each different species of molecule present. What makes this view at least a possible one is the fact that though thermal equilibration over molecular distances may be an extremely rapid process, yet there are other processes, such as chemical reaction and diffusion, which are comparable in rapidity. Thus these processes may be able to 'take advantage' of microscopic temperature fluctuations before thermal conduction has evened them out.

An important fact in connexion with the active transport of water under temperature gradients lies in the quite unexpectedly great effect of small temperature differences. Consider a very simple model. Two open Petri dishes I and II (Fig. 2) contain an aqueous solution of osmotic pressure π. Initially both are at the same temperature, and we can suppose them enclosed in a larger sealed vessel. If now dish I be raised in temperature from T to $T+\Delta T$, the vapour pressure above it will be increased and water will evaporate from it and condense in dish II, which will become more dilute

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† These two gradients can be included together as a gradient of chemical potential.

in consequence. This process will go on till the dilute and cooler solution in dish II has the same vapour pressure as the stronger and warmer solution in dish I, when the system will come in a sense to a standstill. There will then be a difference in osmotic pressure $\Delta\pi$ between the two solutions, and this difference can broadly be said to balance the temperature difference ΔT. The relation between $\Delta\pi$ and ΔT can easily be shown to be (Spanner, 1952)

$$\frac{\Delta\pi}{\Delta T}=\frac{L}{VT}, \tag{8}$$

where L is the latent heat of vaporization and V is the molar volume of water, i.e. about 18·0 c.c. A simple calculation from this shows that a temperature difference of only $\frac{1}{100}$° C. can cause a pressure difference of over four-fifths of an atmosphere.

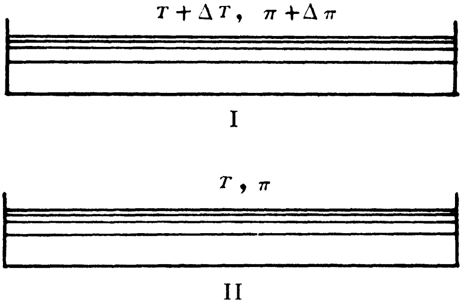

Fig. 2.

A general thermodynamic relation

The simple example just given is a particular case of a general relation governing transport under a temperature gradient. The system we considered consisted of two homogeneous parts I and II separated by a divisional wall represented by the air space. In general this type of system can be represented by such a diagram as Fig. 3. It is called a discontinuous system because its properties change abruptly on crossing the membrane from one side to the other, though within each section all properties are uniform.

Suppose that in such a system (containing for simplicity, water only) a temperature difference ΔT is imposed between the two sides. In general this will cause a movement of water from one side to the other, and provided the walls are solid this movement will build up a pressure difference ΔP which will ultimately bring the flow to a standstill. When this occurs the system is said to be in a 'steady state', and the relation between ΔP and ΔT will be given by the perfectly general equation

$$\frac{\Delta P}{\Delta T}=-\frac{Q^*}{VT}, \tag{9}$$

where V is the volume of unit mass of water and Q^* is a quantity called the heat of transfer'. In a sense therefore equation (9) gives the equivalence of temperature and pressure differences in promoting flow across the membrane; if the right-hand side is large then a very small temperature difference can cause the same rate of flow as a much larger pressure difference. The key to the situation obviously lies in the interpretation of the quantity Q^*, which must now be discussed.

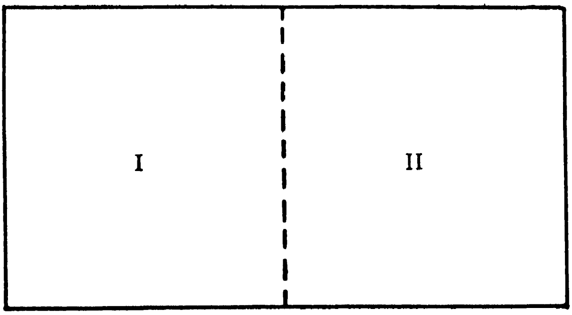

Fig. 3.

III. THE 'HEAT OF TRANSFER' Q^*

Imagine that the whole system represented by Fig. 3 is at a uniform temperature, but that owing to a small pressure difference (ideally an infinitesimal one) water is flowing across the membrane. In general, the process by which the water traverses the membrane will act differentially on the faster and slower molecules so that the water which crosses is not usually a representative sample but contains either a larger, or a smaller, proportion of 'hot' or 'cold' molecules than the bulk. This is very obviously the case in the example previously given, where the membrane is an air space and the process of crossing it involves, first, evaporation and, secondly, condensation. Thus, as the water moves across, it carries a quantity of heat with it, and while compartment I is left cooler, compartment II grows warmer—again a result which is very evident in the evaporation example. Suppose now that as the water flows across the membrane heat is continuously abstracted from compartment II to maintain its temperature constant. Then the quantity of heat so abstracted per unit mass of water flowing is the 'heat of transfer', Q^*. In the earlier example (Fig. 2) it is obviously very nearly the same as the latent heat of evaporation, showing that equation (8) is a particular case of equation (9).

Conditions for large 'heat of transfer'

It can be seen at once that the condition which must be fulfilled if there is to be a definite heat of transfer is that the membrane should act in such a way as to distinguish between 'hot' and 'cold' water molecules. In general, there are two ways in which this can come about, and these two incidentally correspond to the two classical theories of cell permeability. In the first, the membrane may act as a sieve. If the pores of this are coarse, no selective effect is apparent; water flowing down a main does not tend to get hotter as it goes. On the other hand, if the pores are really minute, in fact comparable to the intermolecular distance of water, then the faster molecules penetrate in relatively greater numbers than the slower ones, and a 'heat of transfer' appears. This is particularly easy to show with gases and a fine-grained porous pot. The magnitude of Q^*, however, is small, and even in the most favourable cases it never numerically exceeds $\frac{1}{2}RT$ per mole.

In the second mechanism, the membrane acts as a potential energy barrier. This means that it does not merely act less favourably towards low-energy molecules (as the sieve does); it positively turns them back. Thus in the example of Fig. 2 only those water molecules with energies sufficient to overcome the latent heat forces can escape into the 'membrane'; all with lower energies are held back. From this it can at once be seen that the 'heat of transfer' can be very large in such cases; in fact it is very nearly the same as the height of the potential energy barrier, i.e. as the 'activation energy' required to cross the membrane.

This second type of membrane may take many different forms. It may be simply an air space with transport occurring in the state of vapour; or it may impose chemical reactions as a condition of crossing, as when some gases diffuse across metallic diaphragms. A very important type of membrane involves what can be called 'solution in an uncongenial medium', and it is here that we can probably place the case of water molecules penetrating the lipoid membrane of the protoplast. Still another type of potential energy barrier is the electrical one. This is exemplified by a charged membrane exposed to ions, and illustrates the fact that the barrier can be either positive or negative, i.e. it can either oppose or facilitate crossing. In the latter case the 'heat of transfer' may be negative, since the barrier (which might better be called a 'ditch' in this case) acts to promote the crossing of disproportionate numbers of *low*-speed molecules.

Whatever the mechanism of crossing, however, equation (9) holds, exemplifying in this respect the typical nature of a thermodynamic result. However, before any practical use can be made of this relation it is obviously necessary to know the order of magnitude of Q^*. Where the mechanism of 'permeability' is known it may be possible to arrive at this from consideration of existing and well-known data, as in the case of evaporation, where it is equal roughly to the latent heat. As a matter of fact, as mentioned earlier, in all cases Q^* is equal very nearly to the height of the potential energy barrier (if this is large); but as this is very rarely known, some other way of evaluating it must be found.

IV. MEASUREMENT OF THE 'HEAT OF TRANSFER'

Fortunately, Q^* can be calculated very simply from a knowledge of the temperature dependence of the permeability. As a semi-empirical but fairly exact relation Arrhenius showed that the equation

$$\frac{\partial}{\partial T}\ln(\text{rate}) = \frac{E}{RT^2} \tag{10}$$

can be used to describe the variation of rate processes with temperature, E being the 'activation energy' of the process. Originally this equation was applied to chemical reactions, but it can be used for transport and other processes as well. It has been regarded till fairly recently† as only having a partial justification in theory, but it appears (Appendix) that it can be treated as quite exact if E, the 'activation energy', is replaced with Q^*, the heat of transfer. When this is done and the ordinary permeability μ is introduced into the equation we get the exact result

$$\frac{\partial}{\partial T}\ln\left(\frac{\mu T}{V}\right) = \frac{Q^*}{RT^2}, \tag{11}$$

where V is the partial molar volume of water. Since V is nearly constant it can be omitted, and assuming that Q^* does not vary greatly with temperature equation (11) becomes on integration

$$\ln\left(\frac{\mu_2 T_2}{\mu_1 T_1}\right) = \frac{Q^*}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right). \tag{12}$$

Writing $T_1 \approx T_2 = T$, $T_2 - T_1 = 10$ and introducing the temperature coefficient Q_{10} of permeability we get the final result

$$\frac{10Q^*}{RT^2} \approx \ln(1{\cdot}034\,Q_{10}) \tag{13}$$

at ordinary temperatures. This result gives us a simple way of evaluating the heat of transfer across the cell membrane.

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† The theory of absolute reaction rates developed by Eyring and others since 1935 gives, of course, a very satisfactory basis for this equation.

V. ACTIVE TRANSPORT OF WATER ACROSS THE CELL MEMBRANE

Broadly speaking, the cell can be regarded as a discontinuous system of the type described, with an internal watery phase separated from an external one by a thin lipoid layer constituting a potential energy barrier. The height of this barrier probably varies considerably, and sometimes the membrane may even act as a molecular sieve. However, assuming a typical value for the temperature coefficient of permeability it is possible to make an estimate of the 'thermomolecular pressure effect', as $\Delta P/\Delta T$ in the steady state is called. For plant cells a fairly typical value for the Q_{10} for water would seem to be about 2·6 (Davson & Danielli, 1943), and using equation (13) this gives the 'heat of transfer' as

$$Q^* = \frac{1{\cdot}986 \times 293^2}{10} \ln(1{\cdot}034 \times 2{\cdot}6)$$

$$= 16{,}900 \text{ calories per mole.}$$

Substituting this value in equation (9) the pressure effect is seen to be

$$\frac{\Delta P}{\Delta T} = -\frac{16{,}900 \times 41{\cdot}3}{18{\cdot}0 \times 293},$$

$$= -132 \text{ atmospheres per degree C.,}$$

the negative sign indicating that the pressure develops on the low-temperature side. This is an astonishingly large value; it means that a temperature difference of only $\frac{1}{100}$° C. can cause movement of water at the same rate as a pressure difference of well over an atmosphere. Before passing to the question, however, of whether a thermomolecular mechanism can be of any practical importance in the life of the organism, it is necessary to consider two objections which raise rather serious difficulties.

Temperature gradients in the cell

The first difficulty arises from the extreme thinness of the plasma membrane. There is a considerable amount of evidence (see, for instance, Davson & Danielli, 1943) that the plasma membrane is normally of the order of 10^{-6} cm. in thickness. Now a temperature difference of $\frac{1}{100}$° C. over this distance means a temperature gradient of $10^{-2}/10^{-6} = 10{,}000$ ° C./cm., which is hardly of the order of magnitude to be expected in living cells; about one-thousandth of this value would seem to be more within the realm of possibility. However, this objection can be partially met in three ways. The thermal conductivity of a typical oil such as olive oil is about one-third of that of water. Further, when molecules are definitely oriented, as in crystals, the conductivity may be widely different in different directions, a factor of even 5 or 6 being sometimes reached. Now the lipoid molecules in the membrane are arranged more or less parallel, and it is not inconceivable that this arrangement might considerably influence the heat conductivity across the layer. If this is so the conductivity of the membrane might be less than that of water by a factor as low as one-tenth or even less. This, however, only goes a small way towards removing the objection. A second possibility lies in the fact that over a potential energy barrier the flow of heat is impeded as well as the flow of matter, and in a diffuse gaseous system the resistance to heat flow can easily be augmented by such enormous factors as 10^{10} or more. This arises from the fact that in diffuse gases heat flow is by diffusion of molecules, whereas in solids collision is the mechanism and no diffusion at all need take place. Liquids come in between, and heat conduction is partly by one means and partly by the other. To what extent therefore thermal flow is hindered by the fact that the plasma membrane constitutes an energy barrier is not apparent, but at least it is a factor to be taken into account, and it may possibly be very important. Where a whole tissue or several layers of cells is concerned of course the necessary temperature differential will be divided between all the plasma membranes, and this will naturally reduce the *gradients* over them; the same is true where the cell seems to possess multiple membranes, as reported recently by Sjöstrand (1953) for mouse pancreas and kidney cells.

Finally, it may be observed that where the temperature gradient is excessively high the ordinary law of thermal conduction will break down, just as the analogous laws for diffusion and electrical conduction do.

It is suggested that these three considerations, taken together, may conceivably raise the resistance to heat flow across the plasma membrane to several hundred, and perhaps even a thousand, times its value for a comparable thickness of bulk aqueous phase, and this will of course correspondingly sharpen the temperature differential over it.

The heat flow accompanying transport

The second objection is an equally serious one. It will readily be appreciated that while transport is actually proceeding (as opposed to the steady state in which it has ceased) very large quantities of heat energy will be passing across the barrier, since all the molecules crossing will be high-velocity ones. As a matter of fact, to a first approximation, the amount of heat flowing across per unit amount of water will be the heat of transfer, Q^*. At this rate a man would have to consume a remarkable weight of potatoes merely to keep his kidneys functioning! In the course of an hour between 3 and 4 l. of water are actively reabsorbed by the kidneys, and if the heat of transfer was only 6900 cal./mole (corresponding to a Q_{10} of 1·5) the heat flow would be 1,340,000 cal., requiring the complete oxidation of no less than 360 g. of glucose per hour, or 19 lb. per day! Fortunately, the body functions more efficiently than this, but it looks on the surface as if any thermomolecular mechanism is ruled out at once. The difficulty arises from the fact that this mechanism implies that the cell membrane functions as a heat engine, and as one working over the extremely small temperature range ΔT. This at once limits the thermodynamic efficiency of the process, as Carnot showed, to the excessively small value $\Delta T/T$, and consequently any direct provision of heat by chemical reactions is inconceivable as a significant contribution. However, an interesting possibility remains. If a heat engine is very inefficient because it works over a small temperature range, it is correspondingly highly efficient when it works in reverse as a heat pump over the same range. In other words, the amount of heat it transfers can be an enormous multiple of the free energy it consumes. Such a reversed mechanism can be imagined in the cell or organ. A very simple case is ordinary osmosis. When a *Paramoecium*, for instance, draws in water osmotically from its environment this entering water will carry a large amount of heat in with it, an amount measured by Q^* for the plasma membrane. There is energetically no reason why this heat should not be sufficient to expel water by a thermomolecular process into the contractile vacuole, whose membrane, conceivably, has a lower value of Q^*. Ultimately, of course, some source of free energy is required, and this may operate by maintaining the required osmotic pressure difference at the points of water entry and exit. In the case of the kidney the possibilities are somewhat similar. Pressure filtration in the Bowman's capsule will hardly imply any considerable flow of heat, as the membrane almost certainly acts as a fairly coarse sieve; but in the proximal convoluted tubules where apparently glucose is reabsorbed it is not at all impossible to conceive that the development of osmotic activity might draw in water from the capillaries across a high energy barrier, the heat provided being later used, perhaps in the loop of Henle, to sustain a reversed flow into the capillaries through an energy barrier substantially lower. Such a process, involving first a heat pump and then a heat-engine activity, might possess quite an appreciable thermodynamic efficiency. At least it appears to be a possibility deserving of attention.

VI. CONCLUSION

The theory outlined in this paper has probably its more important potential application to water movement, though it can also be applied to the active transport of solutes such as sugars and ions. However, in the case of these there seem several much more promising suggestions now being debated, and only in the case of water does it seem likely that none of the existing mechanisms is adequate to meet the requirements. What, in effect, the present theory does is to show that under certain conditions a temperature differential can bring about results hitherto associated only with a pressure difference. Thus the cells of a green alga spending its whole life in fresh water maintain themselves in equilibrium so far as water content is concerned only because they possess a firm wall which is capable of sustaining a considerable internal pressure. This possibility is normally denied to the single-celled organisms of the animal kingdom, and they are consequently faced with the problem of actively excreting water to counterbalance that entering by osmosis. It might, of course, prove to be the case that they excrete actively not water, but ions or other solutes, the water being merely drawn out after them passively by ordinary osmosis. This can only be decided by analysis of the fluid excreted, and it certainly remains an attractive theory, especially in the light of the suggestions put forward a year or two ago by Goldacre (1952) on protein contractility as a basis for osmotic work. However, living systems are amazingly complex, and it is hardly a flight of fancy to suppose that the ultimate explanation of the phenomena of active water movement will be found not in one, but in a combination of several physical processes.

APPENDIX

On the thermodynamic theory

The following is a brief account of the derivation of the fundamental equation. It follows the treatment given by de Groot (1951) and illustrates the method of the Thermodynamics of Irreversible Processes.

Consider the simple system discussed earlier (Fig. 3), water being the only component present and all intensive properties, such as temperature and pressure, being uniform throughout each sub-section. Let superscripts I, II denote the two sections. Further, let subscripts i, e denote increments of an extensive quantity gained internally (i.e. from the other subsection) or externally (from the surroundings). Then it is required to find an expression giving the rate of increase (σ) of the entropy of the system supposing it to be held completely isolated from its surroundings. To do this it is convenient to consider the system as merely *closed*, but not *isolated*; and to separate the expression for its rate of entropy increase into two parts, one representing the entropy gained from the surroundings, the other the entropy produced internally. The latter will be the value of σ, the quantity required. The further development of the theory will then require the use of Onsager's Theorem.

If m^{I}, m^{II} are the masses of water on the two sides of the membrane, the Law of Conservation of Mass gives

$$m^{\mathrm{I}}+m^{\mathrm{II}}=\text{constant},$$

or

$$dm^{\mathrm{I}}+dm^{\mathrm{II}}=0. \tag{1}$$

Further, if U is the internal energy we have the analogous relation

$$d_iU^{\mathrm{I}}+d_iU^{\mathrm{II}}=0. \tag{2}$$

Finally, if the whole system is changing slowly enough each of the sides can be regarded as an open system in internal equilibrium, and we can apply to it the equation of Gibbs:

$$dU=TdS-PdV+\mu\,dm. \tag{3}$$

The validity of this procedure will naturally depend on the transport processes in operation not being of too rapid a nature; in practical cases, however, equation (3) will hold to a very good degree of approximation. Applying it to each side in turn we get

$$dU^{\mathrm{I}}=T^{\mathrm{I}}dS^{\mathrm{I}}-P^{\mathrm{I}}dV^{\mathrm{I}}+\mu^{\mathrm{I}}dm^{\mathrm{I}}, \tag{4}$$

$$dU^{\mathrm{II}}=T^{\mathrm{II}}dS^{\mathrm{II}}-P^{\mathrm{II}}dV^{\mathrm{II}}+\mu^{\mathrm{II}}dm^{\mathrm{II}}. \tag{5}$$

Rearranging these and adding,

$$\begin{aligned}dS=dS^{\mathrm{I}}+dS^{\mathrm{II}}&=[(d_eU^{\mathrm{I}}+P^{\mathrm{I}}dV^{\mathrm{I}})/T^{\mathrm{I}}+(d_eU^{\mathrm{II}}+P^{\mathrm{II}}dV^{\mathrm{II}})/T^{\mathrm{II}}]\\&\quad+\{d_iU^{\mathrm{I}}/T^{\mathrm{I}}+d_iU^{\mathrm{II}}/T^{\mathrm{II}}-\mu^{\mathrm{I}}dm^{\mathrm{I}}/T^{\mathrm{I}}-\mu^{\mathrm{II}}dm^{\mathrm{II}}/T^{\mathrm{II}}\},\end{aligned} \tag{6}$$

where dU has been split up into d_iU+d_eU. The quantity in square brackets represents the entropy gained from the surroundings[†] (d_eS); that in braces, the entropy (d_iS) generated within the system. Had the system been isolated d_eS would of course have been zero, while d_iS would have been unaffected. It is this latter quantity therefore which is relevant to the Onsager theory.

Eliminating dm^{II} and d_iU^{II} from (6) by means of (1) and (2) and dividing by dt, we get for the rate of entropy production

$$\begin{aligned}\sigma=\frac{d_iS}{dt}&=\frac{d_iU^{\mathrm{I}}}{dt}\left(\frac{1}{T^{\mathrm{I}}}-\frac{1}{T^{\mathrm{II}}}\right)-\frac{dm^{\mathrm{I}}}{dt}\left(\frac{\mu^{\mathrm{I}}}{T^{\mathrm{I}}}-\frac{\mu^{\mathrm{II}}}{T^{\mathrm{II}}}\right)\\&=\frac{d_iU^{\mathrm{I}}}{dt}\frac{\Delta T}{T^2}+\frac{dm^{\mathrm{I}}}{dt}\Delta\left(\frac{\mu}{T}\right),\end{aligned} \tag{7}$$

where the symbol ΔT, for instance, stands for T_2-T_1, and $T_1\approx T_2=T$. Equation (7) has the form mentioned earlier; the 'fluxes' are $J_u=-d_iU^{\mathrm{I}}/dt$,

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[†] The expression $(d_eU^{\mathrm{I}}+P^{\mathrm{I}}dV^{\mathrm{I}})$, for instance, is equal to $q_e{}^{\mathrm{I}}$, the heat absorbed from the surroundings by subsection I. This follows from the first law, $P^{\mathrm{I}}dV^{\mathrm{I}}$ being the work done by subsection I in expanding against the surroundings.

the flow of energy from I to II, and $J_w = -dm^1/dt$, the flow of water. The appropriate 'forces' are $X_v = -\Delta T/T_2$ for the flow of energy, and $X_u = -\Delta(\mu/T)$ for the flow of water.

The theory now relates the J's and X's by means of rather general equations analogous to 'Ohm's Law'. This leads to the expressions

$$J_u = L_{uu}X_u + L_{uw}X_w, \tag{8}$$

$$J_w = L_{wu}X_u + L_{ww}X_w, \tag{9}$$

where the L's are conductance coefficients which will depend on the size and geometry of the system and on the nature of the divisional membrane. However, Onsager's theorem states that provided forces and fluxes are measured in the way described the general result always holds that

$$L_{uw} = L_{wu}. \tag{10}$$

This is a result of the Principle of Microscopic Reversibility, according to which reversal of the motions of all the particles in an isolated system would simply cause the system to retrace its former history.

Before proceeding with the theory it is convenient to replace the flow of energy J_u with the flow of heat, J_Q. In a rough way it can be seen that the energy carried from I to II per unit mass of water will be made up of, first, a term depending on the internal energy u per unit mass of water; secondly, an amount of work represented by the product of the volume of water per unit mass (v) and the pressure under which it flows; and thirdly, a quantity of heat. The first two terms make up the heat function per unit mass (h), since

$$h = u + Pv. \tag{11}$$

This leads to the suggestion that we define the heat flow as

$$J_Q = J_u - hJ_w. \tag{12}$$

The quantity h will differ for the two sides of the system, but since the difference is small this will be of no account. It simply underlines the fact that the theory in any case is strictly exact only for infinitesimal departures from equilibrium.

If we introduce J_Q from (12) into (7) we find that the force X_w is altered. This illustrates the ambiguity mentioned earlier when we were discussing the distinction between active and passive movements; the force X_w which is the passive agent for water movement depends on whether we take J_u or J_Q as the other flux. However, it seems logical to take J_Q instead of J_u, since not only is the flow of heat independent of the flow of matter in a sense in which the flow J_u is not, but the choice of J_Q leads to values of the forces which are uniquely defined; that is, they have no unknown additive constant as h or μ, for instance, have.

Substituting for J_u from (12) into (7) the new forces become

$$X_Q = -\frac{\Delta T}{T^2}, \tag{13}$$

$$X_w = -h\frac{\Delta T}{T^2} - \Delta\left(\frac{\mu}{T}\right)$$

$$= -\frac{v\Delta P}{T}. \tag{14}$$

The latter result follows from the fact that $\mu = h - Ts$, where s is the entropy per unit mass; also, since we are dealing with a single substance μ is a function of P and T only. Thus, considering a fixed amount of water we have the Gibbs equation

$$dG = V\,dP - S\,dT, \tag{15}$$

which, on dividing by the total mass and writing Δ instead of d, gives

$$\Delta\mu = v\Delta P - s\Delta T. \tag{16}$$

The 'Ohm's Law' equations can now be written in the form

$$J_Q = L_{QQ}\left(-\frac{\Delta T}{T^2}\right) + L_{Qw}\left(-\frac{v\Delta P}{T}\right), \tag{17}$$

$$J_w = L_{wQ}\left(-\frac{\Delta T}{T^2}\right) + L_{ww}\left(-\frac{v\Delta P}{T}\right), \tag{18}$$

with

$$L_{wQ} = L_{Qw}. \tag{19}$$

The condition for the steady state is found by writing $J_w = 0$. This gives, on rearrangement,

$$\frac{\Delta P}{\Delta T} = -\frac{L_{wQ}}{L_{ww}}\frac{1}{vT}. \tag{20}$$

But by definition, if ΔT is put equal to zero the heat flow per unit of mass (J_Q/J_w) is the heat of transfer Q^*. This gives

$$Q^* = \frac{L_{Qw}}{L_{ww}}. \tag{21}$$

Introducing (21) with (19) into (20) it follows that

$$\frac{\Delta P}{\Delta T} = -\frac{Q^*}{vT}. \tag{22}$$

The temperature dependence of the permeability

Let ν^{I}, ν^{II} be the rates at which molecules of water are passing across the membrane from the two sides respectively. At equilibrium ν^{I} and ν^{II} will be equal, and this will also be true for the steady state.

Starting with the whole system in equilibrium, imagine the temperature and pressure of section I to be raised by ΔT and ΔP respectively. Then the condition that must be fulfilled if the net flow of water is to remain zero is

$$\Delta \nu^{\mathrm{I}} = 0. \tag{23}$$

Treating ν as a function of T and P this becomes

$$\frac{\partial \nu}{\partial T}\Delta T + \frac{\partial \nu}{\partial P}\Delta P = 0, \tag{24}$$

or, more explicitly,

$$\frac{\Delta P}{\Delta T} = -\frac{\partial \nu}{\partial T}\bigg/\frac{\partial \nu}{\partial P} \tag{25}$$

$$= -\frac{\partial (\ln \nu)}{\partial T}\bigg/\frac{\partial (\ln \nu)}{\partial P}. \tag{26}$$

Now it can be shown that ν is proportional to the vapour pressure p. Hence

$$\frac{\partial (\ln \nu)}{\partial P} = \frac{\partial (\ln p)}{\partial P} = \frac{v}{RT}, \tag{27}$$

by a well-known thermodynamic relation, v being the volume of unit mass of water.

But from (26) we have

$$\frac{\partial (\ln \nu)}{\partial T} = -\frac{\Delta P}{\Delta T}\frac{\partial (\ln \nu)}{\partial P}, \tag{28}$$

or introducing (22) and (27),

$$\frac{\partial (\ln \nu)}{\partial T} = \frac{Q^*}{vT}\frac{v}{RT}$$

$$= \frac{Q^*}{RT^2}, \tag{29}$$

which is the familiar Arrhenius type of equation for the temperature variation of a rate.

The ordinary permeability μ is the net rate of flow of water under unit pressure difference and zero temperature difference, i.e.

$$\mu = \left(\frac{\partial \nu}{\partial P}\right)_T. \tag{30}$$

Using (27) it is possible to write

$$\mu = \frac{\nu v}{RT},$$

or

$$\nu = \frac{\mu RT}{v}. \tag{31}$$

Thus from (29)

$$\frac{\partial}{\partial T}\ln\left(\frac{\mu T}{v}\right)=\frac{Q^*}{RT^2}, \tag{32}$$

or since v will be nearly constant,

$$\frac{\partial}{\partial T}\ln(\mu T)=\frac{Q^*}{RT^2}, \tag{33}$$

which is the result sought.

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WATER TRANSPORT IN INSECTS

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I. INTRODUCTION

Among the factors contributing to the success of the Insecta—animals of a comparatively small order of size—the evolution of a waterproof cuticle has been considered of prime importance. Consequently, water exchange between the atmosphere and an insect or its egg has received considerable attention. Other groups of animals have become established on land, either by living in the nearly saturated atmosphere of soil or in other specialized regions of high humidity. The higher vertebrates do not hold to this rule, but at once the order of size becomes apparent. A medium-sized mammal has a surface-area/volume ratio of the order 0·5 sq.cm./c.c. It can survive comparatively high evaporation rates before it need replenish its water supplies. A typical insect egg has by comparison a ratio of 50 sq.cm./c.c., and normally it has no means of replenishing its water from the environment. The active insect is only a factor of ten better off than its egg, and a waterproof cuticle is clearly a necessity.

Wax layers

The principal device which has been evolved by the insect to resist water loss is now well established; it is a layer of orientated lipoid near the surface of its cuticle, or forming one of the layers of its egg-shell (Ramsay, 1935; Wigglesworth, 1945; Beament, 1945, 1947; etc.). The degree of efficiency which can be achieved by wax systems is exemplified by the egg of the mite *Metatetranychus ulmi* (Beament, 1951). The over-wintering egg has a diameter of only 0·14 mm., and its surface-area/volume ratio is 500 sq.cm./c.c. It has hatched successfully after being held at room temperature in an atmosphere of 70% R.H. for a year. Other than its two wax layers (one of which does not cover the whole shell surface) this egg has only a keratin-like membrane and a sticky cement to protect it, the layers together being 4μ thick; it can survive a loss of only 5% of its total water content and has no means, so far as can be demonstrated, of taking up water, even from liquid water in contact with it. If the inner wax layer is incomplete, it dries up in 70% R.H. in a matter of minutes.

The problem of conserving water by 'passive' means has thus been overcome, and very efficiently; outside the insects proper, the wax-layer mechanism is found in ticks (Lees, 1946; Lees & Beament, 1948), mites (Beament, 1953), and probably in spiders (Edney, 1953), though not apparently in myriapods (Cloudsley-Thompson, 1950); there is evidence for believing that the cocoon of the lung fish, *Protopterus* (Beament, 1953), uses a similar mechanism. Undoubtedly a parallel system occurs in the leaf waxes of plants (Stelwaag, 1924; Fogg, 1948; Piper, Chibnall & Williams, 1934).

At first sight, the insect has created two problems for itself by producing this impermeable cuticle, for the very existence of the wax layer may well prevent the absorption of water in the environmental atmosphere, or of liquid water in contact, when such an opportunity presents itself. We must neglect here the osmotic problems of a fresh-water insect with such a cuticle; at the present time no one has demonstrated the existence of wax layers in aquatic insects. Evidence from work on water exchange through the anal papillae of mosquitoes (Wigglesworth, 1933*a*; Beadle, 1939) suggests that such surfaces are much more permeable than we would expect if a wax layer were present. But one can hardly envisage a humidity receptor, or a chemo-sensory organ, on a terrestrial insect, which could function efficiently if covered with wax. It seems very probable that certain surface areas of insects will be left unwaxed to allow of their specialized functions. A general consideration of the physiology of aquatic insects, however, must make it difficult to believe that the whole of the cuticle is freely permeable to water, and a wax layer would surely be the most obvious means of protection against invasion by water.

Water uptake

Because of claims for such a degree of water impermeability, a degree of impermeability which has been repeatedly demonstrated in experiments on isolated cuticle and artificial membranes (Beament, 1945, etc.) to be inherent in the non-living system of the cuticle or shell, it is the more remarkable that certain insects and eggs can take up water from the outside atmosphere, from humidities well below those which would be in equilibrium with the blood fluids. Lees (1946, 1948) shows that the tick, *Ixodes*, can take up water down to 88% R.H., Mellanby (1932) that the mealworm can do so from 90% R.H. and Edney (1945) claims that the flea pre-pupa may do so from humidities as low as 50%. In all these examples, the blood-equilibrium humidity is of the order of 99% R.H. Whether this is a relevant figure, and how it compares with the humidity equivalent of the epidermal cells themselves, must be considered later.

Before discussing the nature of any active water-uptake mechanism, the physico-chemical properties of these natural membranes should be fully understood. We must also distinguish carefully between 'active' and 'controlled' water exchange. In many insect eggs—for example, those of *Melanoplus* (Slifer, 1938), *Locustana* (Matthée, 1951)—the shell is impervious to desiccation for considerable periods of time; when eggs in diapause are placed in liquid water, they do not take up appreciable amounts. Once diapause is broken, however, the egg responds rapidly to the existence of liquid on its surface, and absorbs it rapidly, increasing in weight by as much as 15%. But Matthée has shown that water uptake depends on the availability of oxygen, so that the process must be regarded as 'active' at least in the sense that respiring cells are necessary. It must be emphasized that these eggs are very waterproof up to the time of immersion in water, even though they have come out of diapause; it is even more striking that they apparently cannot take up water from saturated air, though they will do so from aqueous solutions having high osmotic pressures.

Once these eggs start to take up water, the rate of transfusion is so much greater than the apparent permeability of the shell-wax layer when the egg is desiccated in diapause that one might be led to postulate a process in which the cells had very remarkable powers of absorption. But if the water-uptake process is interrupted, desiccation experiments at this time indicate a real, though not necessarily permanent, change in the physical permeability in both directions. This evidence could only be challenged by experiments on inert shell membranes isolated at the relevant times. Hence, here, an undoubtedly 'active' uptake of water is at least accompanied by suspected changes in the inert layers. Now the process of water uptake might be considered as partially osmotic, though this would continue in the absence of oxygen. But apart from the demonstrated ability of eggs to obtain water from comparatively strong solutions, osmosis would rapidly lead to bursting, and this is a very rare event in nature in *Locustana*. The living material must be just as capable of arresting the inflow of water, regardless of existing osmotic gradients, as it is of initiating and maintaining the flow, and we can only conclude that an active non-osmotic process is present. The apparent partial destruction of a wax layer is an entirely different problem; towards explaining this, many suggestions could be made, but when it is followed by repair, perhaps in the presence of water flowing inwards, we are in greater difficulties. Again, we must know the details of the inert changes before we can assess the necessary 'vital' activities which could control the water exchange.

Water exchange in the egg of the garden chafer, *Phyllopertha horticola* (Laughlin, 1953; and unpublished observations which he has kindly permitted me to report), follows a different pattern. This egg does not go into diapause, but in the middle of a comparatively short period of embryonic development there occurs a space of 5 days during which the egg takes up a considerable amount of water. The uptake is accompanied by an increased rate of loss in dry air, ceasing at the end of the water-absorbing phase. Throughout the life of the egg, the desiccation rate in dry air is so much greater than that of typical insect cuticle that one would hesitate to attribute the impermeability of the shell to a wax layer, and no such layer has been demonstrated. To what extent the uptake phase represents an 'active' process is not known, but a contributory factor could be a 'control' of permeability in the form of reversible changes in the shell material.The increased desiccation rate during the uptake phase might yet be greater, but for 'active' secretion on the part of the living material, resisting water loss by desiccation. Suggestions have been made (Edney, 1953, etc.) that such a process could account for the difference in water loss between dead and living animals, and is discussed later. Here, it is much more important to realize that these eggs can successfully complete their whole embryonic period when floating in distilled water. One cannot neglect osmosis when discussing water-uptake mechanisms, and one cannot easily foresee, without the existence of a wax layer, a mechanism by which this living material could tolerate an environment of distilled water in the early and late periods of its existence.

When we compare this egg with that of the cricket, *Gryllulus* (Browning, 1953; and unpublished observations which he has kindly permitted me to report), there is an outstanding difference. The life history of the egg shows a middle water-uptake phase, but the apparent desiccation rate during this period does *not* change materially. This might suggest, even more strongly, the idea of an 'active' mechanism which could both absorb water and oppose loss during desiccation, though it is somewhat remarkable that the combined increase in permeability and active process should give similar water loss as occurs before and after the active phase. It is perhaps more difficult to appreciate what is going on in these eggs, for while, in the chafer, the shell shows every sign of being elastically expanded during water uptake, there is no indication of high internal hydrostatic pressures in the cricket egg at any time in its life—though it will develop and hatch entirely submerged in distilled water and has never been observed to burst due to osmosis. One can hardly suppose that the shell is completely permeable to ions, so that, again, the living egg can in some way negate osmotic forces.

Respiratory envelopes

Wigglesworth & Beament (1950) have shown that a large number of insect eggs have a complete air-sponge layer round the yolk, incorporated into the shell and allowing respiratory exchange over a large area. While this layer does not prevent the existence of continuous solid material from the outside to the inside of the shell, the water-path at this layer is undoubtedly considerably restricted, and largely replaced by a probably slower system of gas diffusion across the sponge.

Water uptake in animals during post-embryonic life

The intimacy of the epidermal cells with the cuticle, and the fact that these cells are directly bathed in blood fluids, makes it difficult to believe that there could be a permanent large discrepancy between the osmotic factors of the two tissues, unless a very large amount of energy is constantly expended to achieve this. Yet the blood is in equilibrium with 99% R.H., whereas in *Tenebrio* (Melanby, 1932), *Xenopsylla* (Edney, 1945) and *Ixodes* (Lees, 1946, 1948) the cuticle can obtain water from much lower humidities.

There are a number of scattered phenomena which must be considered. We are not sure that the water uptake is entirely through the cuticle, i.e. through the externally exposed integument. It may, to an important extent, involve the tracheal system, at the inner end of which another form of water exchange takes place (Wigglesworth, 1933, etc.), and where, under certain physiological conditions, water may be withdrawn and replaced in the tracheole capillaries. But the tracheal system, or at least the inner part of it not subjected to mechanical aeration, is understood to be filled with water-saturated air, and there must be a very water-permeable membrane amounting to a free water surface, present in the tracheal system to achieve this. The main tracheal system is derived from intuckings of the integument, and although it is morphologically very different from the cuticle, there is reason to suppose that it is secreted as the same fundamental procession of chemical entities as compose the multiple laminae of the cuticle: that it has a waxy waterproofing layer on it. A small but interesting observation, which reinforces this idea, can be made when flooding the tracheal system with aerated water. It is a well-known physico-chemical demonstration that air bubbles appear from aerated water when placed in a waxed beaker, but not against chemically clean glass. The appearance of air under these circumstances in the tracheal trunks is dramatic, and more rapid than against the outer cuticular surface. This would seem to mean that in the tracheal lining there is a more hydrofuge surface than on the cuticle, and, possibly, that the cement which overlies the wax layer of typical cuticle (Wigglesworth, 1947; Way, 1950; etc.) is not present in the tracheae.

Hence, so far as water exchange is concerned, we must regard the main tracheal surface as being similar to cuticle, until evidence to the contrary is presented; the tracheole ending, where fluid is visibly absorbed and resecreted, may be a different type of active exchange with the outside world. But the tracheal system is traditionally the main source of water loss from the insect. Clearly the spiracular closing mechanism has been evolved to reduce water loss to a minimum; it has been shown to act (Wigglesworth, 1935) in response to the oxygen requirement and carbon dioxide accumulation of the animal, and not to the saturation deficiency to which the animal is exposed. It would thus seem most unlikely that the tracheole—a source of great water loss—would be the main site of active water uptake, especially when active uptake is apparently so rare a phenomenon, in comparison with the widespread distribution of fluid movement in tracheoles. We must therefore look to the main cuticular and tracheal surfaces for the site of active water exchange.

Water uptake and cuticle damage

Lees (1947) has shown that the ixodid tick is made incapable of taking up water from surroundings at humidities lower than the equilibrium value of its blood fluids, if the epidermis is 'wounded'. Even minute abrasion of the epicuticle, probably only affecting the cuticular wax, the underlying tanned epicuticle and the tips of the pore canals, is sufficient to prevent active uptake immediately.

Now abrasion of this kind is known to elicit a typical wound-healing activity of the underlying epidermal cells (see Wigglesworth, 1940), so that the physiological activity of the epidermis can be said to have been disturbed. Wigglesworth has repeatedly pointed out that the epidermis of an insect is an entity, so that such disturbance of one part of its components might be taken to imply that all of it will behave abnormally. However, the tick recovers its ability to take up water against an apparent gradient of humidity, and this recovery is accompanied by the repair of the cuticular wax layer. Lees shows clearly that the recommencement of the water-uptake process precedes the complete repair of the wax, but it is quite possible that at least a monolayer of wax has been laid down over the whole of the denuded area before uptake recommences. That water uptake can in the later stages accompany repair would would seem to indicate that it is the disruption of the complete wax layer, and not the repair activity, which prevents the uptake process.

Water loss from dead insects

Parallel with these phenomena are the reports that there may be differences between the rate of water loss of some dead and living individuals of the same species. Wigglesworth, in his extensive examination of the cuticle in 1945, states that his figures for water loss are those for dead insects, but providing that the spiracles are blocked, there is no difference between water loss from dead and living animals. He includes the mealworm amongst his experimental material; Lees, on the other hand, indicates the reverse phenomenon in his ticks; individuals desiccated at a time when they could secrete water against a low humidity, lose considerably less water compared with dead individuals. The desiccation rate of the sensescent adult approaches that of the dead animal, and the aged tick loses its ability to take up water from near saturated atmospheres. More recently, Edney (1953), working with spiders (in which he proposes the presence of a typical wax layer), demonstrated not only a slower rate of water loss in the living, as opposed to dead animal, but as between cyanide-killed spiders and those very recently killed by temperature in the course of his temperature/evaporation experiments. He suggests that when a spider is 'dead' as judged by mechanical response, its epidermis is still alive and may oppose evaporation. Edney does not demonstrate any active water-uptake mechanism in these spiders, but his proposal must be considered along with those previously raised by studying ticks and eggs—can the cell resist desiccation? It does not necessarily follow that a process capable of secreting water inwards by doing work will automatically also decrease the rate of flow outwards when the direction of flow through the membrane is reversed; the two systems are not without their physico-chemical differences.

The mention of reversal of flow through cuticular membranes at once introduces the much-discussed phenomenon, usually called the 'asymmetry' of insect cuticle. The confusion which exists over this has resulted in some curious ideas on water exchange through the cuticle, and a real understanding of this asymmetry is all part of the original premise: that one must know the physico-chemical properties of the membrane before one can assess or interpret the mechanism of the active processes which occur.

II. MEMBRANE PHENOMENA

Water uptake through a cuticle was elegantly demonstrated by Ramsay (1935) when he showed that a droplet of water, placed on the cuticle of a cockroach, did not evaporate, but was covered by a layer of grease, present in a mobile state on the surface of the animal. While the grease film substantially reduced evaporation from the droplet, the cockroach took in the water from the interface between it and the underlying cuticle. This cuticle is atypical, for few insects are capable of so isolating a droplet on their surface while they absorb it at leisure.

Ramsay apparently assumed that the cuticle underneath the drop was denuded of its grease, but this is not so; if a cuticle is washed for a long time in the surface of running water so that, by surface spreading, material is swept away, most of the cuticular grease is removed (see Rideal, 1926). But the lowermost monolayer, the one which we believe to be most organized and orientated, and which may be strongly linked to the polyphenol tanned layer of the epicuticle, is not so removed. Indeed, while all the evidence (Langmuir, 1925, etc., on films spread on troughs; Beament, 1945, etc., on cuticle models) indicates that this monolayer is principally responsible for the impermeability of isolated cuticle, experiments on the permeability of isolated cuticles make it doubtful if cold chloroform or similar solvents can remove such monolayers, though (see p. 101) wax solvents certainly have a considerable effect on their permeability.* Thus the cockroach is still faced with the problem of taking up the water droplet through the most impermeable layer of its cuticular lipoid.

Asymmetry of membranes

As long ago as 1845, Matteucci & Cima demonstrated that the rate of flow of water through the skin of the frog and of the eel differed with the direction of flow, when the external conditions were reversed. The phenomenon has been shown with the seed coats of plants (Denny, 1917), and Hamburger's (1908) results with synthetic membranes of collodion and chromgelatin first removed the suspicion that the phenomenon was due to a special property of the products of living material. In arthropod cuticle, Hurst (1941) reported of the blowfly *Calliphora* that 'water evaporates through the cuticle...of the larva more than one hundred times as rapidly in the direction lipoid to chitin than in the reverse direction'. In his 1948 paper, Hurst gives a graph showing apparent ratios of asymmetry of the more reasonable order of ten to one. He, nevertheless, claims that the order is such as to suggest an 'all-or-nothing' phenomenon, and outlines a complicated theory of a porous valve structure in the insect cuticle. It must be emphasized that Hurst's method of measurement of permeability was with an osmometer tube, to which the cuticle was attached by a rubber band; the insects were dissected under water (we are not told if they were previously killed, so that epidermal cells may have been living). The cuticular material was completely saturated with water at the start of the

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* I am indebted to Mr M. Holdgate, for permission to refer to some experiments on the contact angle of the cockroach cuticle which support this view.

experiment and the results on which these claims are based were apparently for the first hour of water exchange when the apparatus was placed in a controlled humidity.

Apart from the very great difficulty of obtaining any sort of waterproof seal between cuticle and glass, it is the writer's experience that it is necessary to expose any sort of permeability measuring device to a particular humidity gradient for at least 48 hr. before a stable value is reached. Enormous values of apparent permeability can be obtained when a wet endocuticle, with or without attached cellular debris, is exposed to a dry atmosphere. And since Wigglesworth (1945) and others have demonstrated the dramatic change in permeability produced when minute cuticular abrasion disrupts the wax layer, the only true demonstration of asymmetry is one in which the *same* piece of cuticle in the identical state of preservation is successively exposed to the same humidity gradient.

Readings of permeability are thus only valid in dynamic equilibrium, and it is obviously desirable to reverse the membrane (or gradient) a number of times to be sure that the material has not suffered damage. It is further necessary to run two controls; a free water surface, checking both saturation deficiency and temperature fluctuation, and a blank apparatus to show surface condensation and loss during weighing operations, etc. The water loss of an *intact* piece of cuticle is of the order of 10 mg. or less in 24 hr., and water exchange over the remainder of the apparatus, or fluctuations in saturation deficiency may easily be of the same order, sufficient to mask asymmetry or to double the apparent effect. It is therefore obviously worth while to describe in some detail an apparatus used to obtain the results quoted below.

The design of an experiment on permeability

Apparatus has been evolved from the simple membrane holder described by Beament (1945). It consists (Fig. 1) of a Pyrex water tube, having a projecting flange at one end, and reduced to a narrow bore at the other. A plate of electron metal is placed over the tube, with soft rubber seating on to the glass, and an identical plate is attached below by four brass screws, which also form supports for the whole device. The cuticle is clamped between two electron metal rings, chosen to suit the size of the experimental material, having pressure projections turned on both faces, and these are themselves clamped in a brass cell with screws to provide overall compression. All metal parts are interleaved with washers of reinforced rubber. The unit containing the cuticle sample is thus a robust structure, which can be mounted either way up between the plates of the water tube; the cuticle can be treated in various ways in its holder, while care is taken that it undergoes no mechanical damage at all. The upper end of the water tube is closed by a polythene tube, carrying a short length of fine glass capillary, to allow pressure equilibration between inside and outside, but the minimum of diffusion. The whole construction is designed to have minimum weight (well under 100 g.) so that it can be weighed on a chemical balance.

Sets of cuticles, cast skins, or artificial membranes were mounted in brass cells and stored against use in a desiccator over phosphorus pentoxide. For each experimental determination, six membranes of the type of material being investigated were placed in the holders, three with wax outwards and three reversed. A further holder, with a cell containing a metal disk an eighth of an inch thick in place of the membrane, formed the control, while free evaporation was recorded by a similar apparatus containing a disk of porous pot, with its circumference sealed with thick beeswax. Water was added to a standard height in each tube, capillaries placed over the ends, and the set of holders placed over selected humidity solutions. They were weighed at intervals of 24 hr. until agreement of two successive readings of water loss showed that stable conditions had been reached. The sets of membranes were then reversed, the operation repeated, and in some cases many reversals were carried out. It was hoped

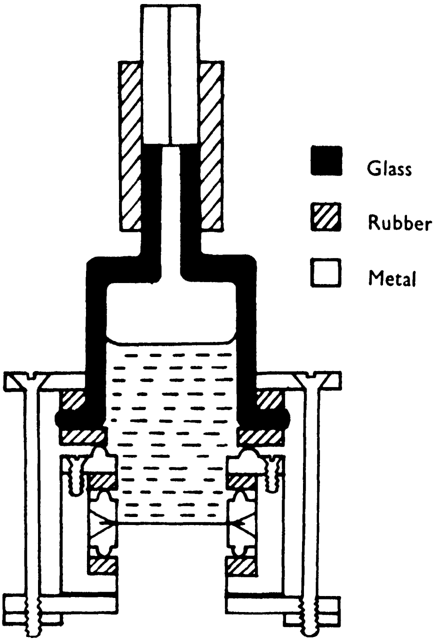

Fig. 1. Sectional drawing of membrane holder for measuring permeabilities.

by this scheme to eliminate sources giving rise to the appearance of asymmetry. Results are given in Table 1.

Table 1. *Abstract from determinations of membrane asymmetry*

Rates expressed in mg./sq.cm./hr.; inner surface against pure water; outer, 2 cm. from phosphorus pentoxide surface in still air; temp. 20° C.; evaporation reference, 24 mg./sq.cm./hr. from free-water surface in membrane position; limit of experiment: asymmetry less than 1:1·09 may not be significant.

Membrane	Treatment	Direction of flow	Rate	Asymmetry
Rhodnius, 5th whole cuticle	—	Endo-epicuticle	0·81	1:1·7
	—	Epi-endocuticle	0·40	
Rhodnius, 5th exuvia	—	Initial exo-epi-cuticle	0·17	1:19
		Average epi-exo-cuticle	3·29	
	4th reversal	Exo-epicuticle	0·57	1:5·5
	Boil in chloroform	Exo-epicuticle	19·3	Not significant
		Epi-exocuticle	19·8	
Periplaneta, late nymphal exuvia	—	Exo-epicuticle	0·42	1:2·3
	—	Epi-exocuticle	0·96	
	Wash in water surface, monolayer of lipoid	Exo-epicuticle	1·73	1:1·2
		Epi-exocuticle	2·06	
	Boil in chloroform	Exo-epicuticle	20·4	Not significant
		Epi-exocuticle	20·1	
Beeswax on parchment	—	Parchment-wax	17·3	1:1·2
		Wax-parchment	20·8	
Beeswax on wax-free cicada wing	—	Wing-wax	4·2	1:2·2
		Wax-wing	9·2	
Rhodnius wax on wax-free cicada wing	Heat to 60° C. dry before measuring at 20° C.	Wing-wax	0·80	1:4
		Wax-wing	3·10	
Beeswax on tanned gelatin standard thick membrane	—	Gelatin-wax	1·97	1:4
		Wax-gelatin	8·12	
	Wax free		15·3	

Discussion of results

The selected membranes are those of (1) *Rhodnius*, an insect whose cuticle is more fully understood than that of any other (Wigglesworth, 1933*b*, 1945, 1947, etc.), (2) *Periplaneta*, whose cuticle is the subject of current research into cuticular grease secretion (Beament, 1951*b*), and (3) 'artificial' cuticles, both of lipoid-free cicada wing covered on one side with beeswax, and of tanned gelatin membranes (prepared as described by Beament, 1945) similarly treated with wax.

All these membranes are asymmetric, though the ratio of permeation in either direction, when subjected to the extreme gradient of pure water/< 5% R.H., does not exceed five to one in any true cuticle; the ratios for artificial systems are lower. There is no suggestion of an 'all-or-nothing' process; rather that the highest ratio is obtained when the membrane in its 'natural' sense has a value of impermeability of the same order as that of an intact insect and therefore, presumably, has the most perfectly organized lipoid layer. Hurst (1948) purports that the asymmetry ratio of blowfly cuticle is not radically altered by chloroform treatment and concludes that the phenomenon of asymmetry cannot be anything to do with the orientated lipoid layer; these results would indicate exactly the reverse. A cockroach cuticle, repeatedly washed in running water, and (see above) therefore with only a monolayer of grease on it, still shows asymmetry of two to one in the best example, and is significantly asymmetric in the worst case.

Comparing the order of asymmetry with the order of permeability in all results, we might conclude that in comparatively permeable membranes there could be pinholes. Whole cuticle preparations have often such perforations where the ducts of dermal glands have been broken off, whereas cast skins, though more delicate, are more likely to be intact, since the lining of the gland is shed with the rest of the outer cuticle. Further, in extreme cases, the swelling of artificial membranes accompanying water uptake could be held to cause some disruption of the continuity of a superficial wax layer. (This phenomenon obviously does not occur in living systems.) But, as outlined below, any tendency for disruption of wax through swelling will produce an effect on permeability tending to reverse the typical asymmetry, and only enhances the values which have been obtained here. The results with exuviae of *Rhodnius* need an extra word of explanation; the permeability in the natural sense does not seem to change during the extensive series of reversals in the same way that values for the reversed sense do. There can be little doubt from our knowledge of the cuticular wax of this insect that it remains substantially unchanged and unaffected by long exposure to water (under its cement layer), and it seems likely that the drop in permeability in one direction only could be due to solvation of hydrophilic material from the inner surface of the skin, during exposure of that side to water.

III. THE PERMEABILITY OF COMPOUND MEMBRANES

The phenomenon of asymmetry in itself is probably of no great consequence to the insect, for, so far as can be seen, no insect is capable of reversing its cuticle at will, and therefore of making use of the phenomenon in a particular circumstance. But an understanding of the causes of asymmetry may give us a better understanding of the whole mechanism of transport through cuticle, considering only the non-living system, and may therefore give us ideas of the way in which the living material may create circumstances in the cuticle, such that water may flow in a particular direction.

The tanned gelatin model

Membranes of tanned gelatin (see Beament, 1945) when placed between water and a dry atmosphere transmit water at 15 mg./sq.cm./hr. When covered with beeswax, on one side to a thickness of 2μ, their permeabilities are respectively 2 and 8 mg./sq.cm./hr. in the directions gelatin/wax and wax/gelatin. (Since the tanning of gelatin is a progressive process which may take years to complete, we should make it clear that the evidence quoted here has all been obtained from one batch of material in a sufficiently short period of time to ensure that the characteristics of the material have remained constant.) If we consider any membrane of uniform constitution, transmitting water at a steady rate X, when placed between two different spacial concentrations of water molecules, we can discuss the dynamic state as follows:

(1) Across the interface between membrane and higher water concentration there will be an overall rate of flow X. This rate will be the resultant effect of a number of forces, such as result from the hygroscopic property of the membrane, suction forces, etc., and saturation deficiency, osmosis, etc., in the reverse direction. For a known rate of flow we can determine a corresponding concentration of water in the membrane surface.

(2) Similarly, there will be a set of forces at the interface between membrane and lower water concentration, and for a flow X there will be a corresponding water concentration in the membrane surface.

(3) At any arbitrary section of the membrane, a consideration of forces across the section must give rise to a flow X. There must be a concentration gradient across the membrane which may not be linear (and will not be so unless the materials obey Fick's law). We may, however, investigate this gradient empirically.

(1) *Water uptake.* A very thin membrane (from the same batch of material as the thick membranes) was used, to ensure minimal difference of concentration between the surface and throughout its thickness. It was desiccated for several days, suspended by fine wire from a torsion balance, and immersed in a beaker of distilled water at 20° C., for periods of 1 min., removed, adhering water immediately blotted off with pads of filter-paper, the balance read, and the operation repeated until the gelatin ceased to take up any further water. Fig. 2*a* shows the rate of uptake across unit surface area, against corresponding concentrations of water. Uptake is inversely proportional to concentration; the rate is very high until the membrane contains some 16% by weight water of the eventual saturation value. There is a uniform slower rate over the range 16–75%, and a final rate of lowest level until saturation is reached.

(2) *Water loss by evaporation.* The same piece of material, fully saturated from liquid water, was then hung from the torsion balance (through a small hole in the lid of a desiccator) over phosphorus pentoxide. Fig. 2*b* shows the rate of evaporation against water concentration in the material, expressed as a percentage of the *saturation water content in liquid water.* The rate of loss, for all values of water content down to 16%, is similar to that of a free liquid-water surface; below 16% it falls regularly with water content.

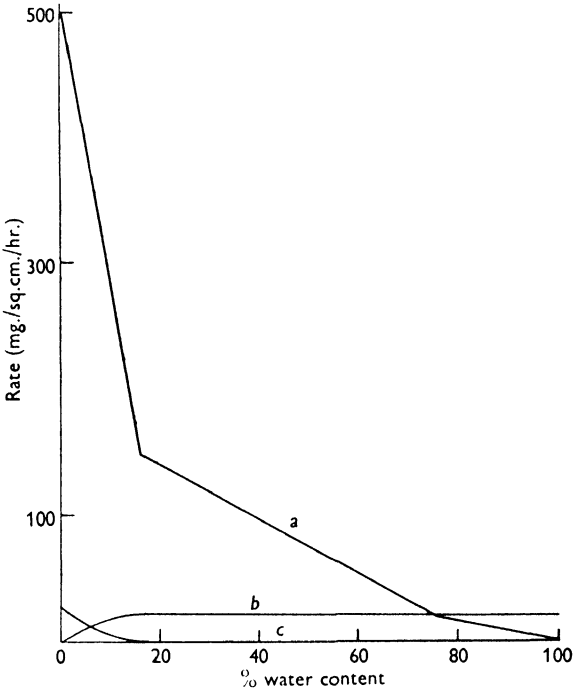

Fig. 2. *a.* Graph showing rate of uptake of a very thin tanned gelatin membrane when in liquid water. *b.* Rate of loss of water when in dry air. *c.* Rate of uptake of water when in 100% R.H.

Water uptake from saturated air was similarly determined. The rate/concentration curve is shown in Fig. 2*c*. The initial rate is about one-twentieth that for corresponding concentrations of water in the gelatin when in contact with *liquid* water. The gelatin comes into some form of equilibrium with the saturated atmosphere at about 16% of liquid saturation content, but when left in the saturated air, small random changes in weight occur over long periods of time, presumably due to the instability of static 'saturated' air.

The gelatin, when in equilibrium with saturated air at 16% water content, will follow Fig. 2*a* when transferred to liquid water. We suggest that the relationship between tanned gelatin and water molecules may be discussed in terms of two factors: a hygroscopic activity, and a 'suction force'. Suction force can only be exercised against water in the liquid phase, and while it accounts for high rates of uptake in liquid water, its contribution to uptake from saturated air is negligible. Nor can suction force retard evaporation from the surface; from Fig. 2*b* retardation of evaporation only occurs by hygroscopic activity when water content falls below some 16%, which is also the figure at which uptake from a saturated atmosphere materially ceases. The state of a membrane which has a greater water content than some 16%, placed in saturated air, is very unstable.

(3) *Water distribution in membranes.* The curves enable us to obtain the water concentration in the surface of a membrane when taking up water from the liquid phase at a known rate, and also the surface content at the other side of the membrane, evaporating at this rate into dry air. The actual percentage of water content of the membrane, when transmitting at a steady rate, is obtained by rapid removal from a membrane holder, blotting, weighing, drying and reweighing. From a consideration of two or more identical membranes which are superimposed (the permeability measured as one thick membrane) and then at once separated and weighed, the linearity of the gradient can be established. Results show that the gradient is substantially linear, within the limits of the experimental method.

Passage of water through wax

A disk of beeswax was subjected to all the experimental conditions imposed above on the tanned gelatin disk. Within the limits of surface condensation, there is no measurable uptake or loss. So far as a composite membrane is concerned, we can conclude that the molecules of wax will not introduce forces tending to move water molecules, but only produce a resistance to water flow. It is doubtful if they will counter-attract evaporational loss at a wax surface. But, in addition to the suggestion that the wax of a double membrane is a high 'resistance', the restriction of flow through the tightly packed molecules of an orientated monolayer must mean that the wax restricts the mean free path of the water molecule to such an extent that it is in the liquid phase when traversing the wax, and *also at* the wax/gelatin (or wax/cuticle) interface.

IV. A PHYSICAL BASIS FOR ASYMMETRY AND WATER UPTAKE

Artificial systems

When a standard tanned gelatin disk is transmitting water from pure liquid to dry air, at 15 mg./sq.cm./hr., the inner and outer surface concentrations (read from the curves) are respectively 80 and 10%, so that a concentration difference of 4·7% gives rise to unit flow. When waxed and transmitting in the direction gelatin to wax, the rate is 2 mg./sq.cm./hr.; the inner concentration of the gelatin is 97%, the drop across the gelatin component must be 2 × 4·7, and in the surface against the wax, 97 − (2 × 4·7), i.e. 87·6%. But water at this interface is still in the liquid phase and remains so until emitted by the wax to the dry air. Therefore there is a suction force at the gelatin/wax boundary tending to retard the outward flow of water molecules. Water in the wax is flowing at the observed rate due to evaporational disturbance of equilibrium at the outer surface, opposed by suction force.

In reversed conditions, a flow of 8 mg./sq.cm./hr. from the gelatin surface by evaporation means a surface concentration of 4·5% water. The concentration difference between the two sides is 8 × 4·7, so that the water concentration at the gelatin/wax interface must be 42·1%. We are still considering water in a liquid phase, so there will be a considerable suction force on the inner surface of the wax, while at the boundary with liquid water there is neither suction force due to the wax nor retention force due to the water reservoir. The rate of flow through the wax must be due to high gelatin suction force.

Now it is very doubtful if we could talk about a 'force of evaporation' nominally measured by the rate of evaporation of a pure water surface into a given saturation deficiency, and equilibrate this with a suction force, measured in the same units of rate of flow across unit area. But in this experimental example, such an equilibration,

$$\frac{24-9{\cdot}5}{95} \mathrel{\hat{=}} \frac{6{\cdot}5}{1} \mathrel{\hat{=}} \frac{\text{evaporation} - \text{suction force at } 87{\cdot}5\,\%}{\text{suction force at } 42{\cdot}5\,\%},$$

gives a reasonable fit with the observed asymmetry ratio. The calculations are supported by measurement of water content of waxed membranes in a steady state of flow; these are in close agreement with the values to be expected from the gradient concentrations.

General principles

It would seem that any membrane, consisting of two laminae, one of which can exercise a considerable suction force on liquid water while the other has very little water affinity, will show the phenomenon of asymmetric water transport as defined in this discussion (see Hartley, 1948; Beament, 1948*a*). But it would seem necessary to add the limitation that in the less hydrophilic component, water must be transported in the liquid phase, otherwise the suction forces which cause this type of asymmetry cannot act. It follows further that the greater the impermeability of the 'resistant' layer, the greater will be the asymmetry. For, in a tanned gelatin model, if the wax could be so impermeable as to reduce water loss to 0·1 mg./sq.cm./hr. (the order of impermeability of typical insect cuticle and shell), then, with water moving in the direction gelatin to wax, the gelatin will be almost saturated throughout, suction force preventing water entering the wax will be negligible, and the measured permeability will approach the theoretical value for a very thin wax layer alone. On reversal, the gelatin will have a very low water content, and at the surface next to the wax will still be so unsaturated as to exercise suction forces corresponding with rates of flow in the 500 mg./sq.cm./hr. region. There is no opposing force on the other side of the wax. One may, without commitment, compare the rates of water movement of free water placed in the situation of the wax in both cases. This is 24:500, and the ratio, 1:21, is very close to the recorded asymmetry of *Rhodnius* exuviae.

Natural systems: cuticle

Under normal circumstances—certainly at the times when it is known that an insect can take up water—there are pore canal tips, filled with living material immediately below the tanned outer lamina of the epicuticle. It is not therefore necessary to consider, in the first instance, the multiple layers of the cuticle. Whether the actual order of suction forces produced by this tanned material is the same as that demonstrated by tanned gelatin must await experimental evidence, but it is a heavily tanned protein and one would expect it to behave very similarly; at least the physico-chemical behaviour of water movement in the epicuticle must be identical to the gelatin-wax model. Further, the material between the living pore canal and the wax is exceedingly thin, possibly less than 1 μ thick, so that a relatively small concentration difference across it would maintain an appreciable flow of water. Hence, to achieve a suction force beneath the wax *and* a gradient, the tanned material next to the cells themselves need be reduced in water content to a very small degree below liquid saturation.

Following on the explanation of asymmetry on the exuviae of *Rhodnius*, it is equally apparent that the smaller ratios found with whole cuticle must be due to the greater thickness of the overall hydrophilic component; this means a much greater difference in concentrations between the sides of the hydrophilic layers, with consequent reduction in the asymmetry of the forces acting on the wax. We have already pointed out that asymmetry in itself is of no great biological consequence to the insect; but some insect egg-shells, such as those of *Rhodnius* when first laid, have their wax as the innermost layer of the membrane system, and are in effect cuticles turned inside out. It is therefore interesting to note that these eggs (Beament, 1948*b*, 1949)—and, one suspects, those of other species—add material to the inside of the shell after oviposition, which turns the system into a 'sandwich', rather than a reversed cuticle.

Water uptake in humidities less than saturation

Here, at the inner surface of the wax, there must be a greater force acting inwards on water molecules than those due to evaporation at the outer surface. But the wax enables the epidermis to make use of suction force in the tanned epicuticle. If, in the round terms of the tanned gelatin model, a relative humidity of 90% produces an outward force proportional to saturation deficiency, i.e. to a free water-surface evaporation-rate of 2 mg./sq.cm./hr., then from Fig. 2*a* it is only necessary to reduce the water content in the underlying component by about 1% to achieve a suction force to resist outward water movement. Seeing the small order of actual uptake rates recorded by Lees (1946) and the thinness of the tanned cuticular component, it seems likely that a decrease in water content of this order might well set up the necessary gradient as well. It is surely within the ability of living cells to regulate the water content of the tanned layer to this extent, and thus to take up water from humidities lower than that in equilibrium with the blood fluids.

When the cuticular wax is abraided, the tick at once loses water into air at 90% R.H. Suction force can no longer act over the abraided area; the denuded cuticle is similar to the gelatin in Fig. 2*b*, where, until the water content is reduced to values of the order of 16% of liquid saturation, there is no retardation of evaporation below the rate given by a free-water surface. It is certainly beyond our idea of vital secretion for the epidermis to halt desiccation by such a reduction in cuticular water. There will be a Brown-Escombe pin-hole effect from the abraided area, and undoubtedly the water loss from this will mask any uptake which might still be going on over the unaffected area, since unimpeded water loss will be so much more rapid than uptake through the considerable resistance of the intact waxy regions.

There is no need to evoke the idea that the physiological disturbances reported in epidermal cells, under abraided regions, have knocked out water-uptake mechanisms. While repair activity goes on for several days, water uptake may recommence after 1 day's repair. But our evidence shows that only a monolayer of wax is necessary to make a cuticle asymmetric: to allow use of suction force on the condensed water in the wax, and therefore to set up the mechanism necessary to reverse the flow through the cuticle. If the mechanism for wax secretion in the cockroach (Beament, 1952) is commonly represented in the arthropods, and freshly secreted wax to repair the abraided area flows over the region in a solvent, then the laying down of a monolayer could be achieved in the first day of repair. Further, the part played by the epidermis in uptake against saturation deficiencies is made clearer when one considers the circumstances in which no uptake can be demonstrated. Lees (1946, 1952), who holds that the pore canals of the engorged tick become progressively filled with solid material, states that the ability to secrete water is lost in engorged animals; Mr M. Locke (in unpublished observations kindly made available to me) has evidence suggesting that the mealworm stops taking up water when the epidermis moves away from the cuticle prior to moulting, and starts again soon after the moult is complete. Obviously the close application of protoplasm to the thin tanned protein of the epicuticle is essential to the process; the cells take up water by regulating the water content of this layer.

Uptake from water in the liquid phase

Mattheé (1951) states that in *Locustana* eggs, liquid water is absorbed—even water from hypertonic solutions—but eggs in this physiological state will not acquire water from saturated atmospheres. The shell material (around the hydropyle in this egg, but the argument holds over the whole surface of other eggs showing similar phenomena) can exercise suction force against water in the liquid phase, but cannot attract water in the vapour phase unless the concentration in the outer shell is drastically reduced. It is obviously well beyond the vital process to achieve such a gradient across the thick shell layers, which would mean an even lower concentration at the inner end of the gradient. When such eggs are desiccated, there must be very considerable suction forces possible, acting outwards, in the inner layers of the shell. Providing there is a wax layer there will be a considerable resistance to be overcome by these suction forces and if there is (as is usual) a proteinaceous layer inside the wax, then the concentration of water here will determine suction forces opposing on the other side of the wax. Thus in the *Rhodnius* egg, when there are two waxy layers, there could very well be a low water content inside the first of these with consequent reduction in forces tending to move water outwards. This idea is supported by the observed dramatic drop in water loss from the *Rhodnius* egg just before blastokinesis (Beament, 1949). One might almost envisage, along these lines, that the secretion, or removal, of layers having high suction forces inside the wax layer in an egg would have a greater importance on regulating water exchange than the secretion and removal of wax layers themselves.

Lees has further shown in the intact tick, in a suitable state for active water uptake, that the rate of uptake is rather more rapid when the animal is placed in liquid water than when it is in a saturated atmosphere. Presumably this is merely due to the greater availability of water molecules at the outer surface. (There is no question of a suction force acting inwards on liquid molecules near the wax.) But when abrasion has removed the tick's ability to obtain water from humidities less than 99% R.H., it can yet take up water at a vast rate when immersed in the liquid, for without its wax the cuticular material can exercise suction force, and there is no wax to provide a high resistance to flow.

Locke (1953) has shown that if, following partial desiccation, mealworms take up water from 93% R.H., they may occasionally reach an equilibrium weight below their starting point. Following this, a period of desiccation for 2 hr., which does not lead to a measurable loss of weight, promotes a further uptake of water, when the animal is then returned to 90% R.H. This is not a matter of restoring an equilibrium water content after desiccation; the new steady level following the second uptake is higher than after the first uptake. But after short, vigorous desiccation, the mealworm will at first lose water from its cuticle layers; an equilibrium with the environment affecting the whole system of cuticle and cells will eventually be set up, but the immediate effect is to produce, by disturbance, a higher suction force against the inside of the wax, with consequent opposition to water loss. When thus transferred to high humidity at once, a considerable uptake rate is promoted, due to physical processes, and apparently the epidermal cells adventitiously absorb this water until a new equilibrium is established. This would suggest that the epidermal cells, at least in mealworms, set up gradients, respond to changes in cuticular water content and accommodate themselves to humidity conditions rather slowly. The total water content of the tanned epicuticle, and the actual amounts of water exchanged under normal circumstances, are very small indeed, and the rates and amounts of exchange during abrasion are so great as to be considered very abnormal circumstances for the cells. While we have concluded that the process of abrasion does not, in itself, knock out the vital basis for secretory uptake, it is still possible that the epidermal cells would show a wound reaction to the vast water exchanges produced by abrasion, regardless of mechanical damage to protoplasmic processes.

Following along the same line of argument, an egg (such as a cricket or chafer egg), though immersed in distilled water, and without such an impermeable shell as would be expected if a wax layer were present, could nevertheless prevent any inflow of water, providing it could maintain in the innermost layer of its shell a completely liquid-saturated layer. This would mean maintaining 100% water, as opposed to something like 99% water, in equilibrium with the osmotic pressure of the underlying cells, though it would still be pure water in both cases. There could, under these conditions, be no water uptake, since there could be no gradient falling towards the inner side. There would, on the other hand, be a dynamic exchange due to random diffusion. (The process could readily be examined by putting eggs in heavy-water solutions, to determine exchange in equilibrium, where no high resistance to flow is expected.) If the living material decreases the water content of the inner shell, water will flow in, as does happen in the mid-embrionic period, and flow will cease so soon as 100% conditions are reasserted.

Two further important points arise when considering eggs. The rate of loss of many eggs in dry air is considerably greater than that through a corresponding area of typical cuticle. It has been assumed on this evidence that such shells would not have a wax layer present. But if such shells are analogous with the physical model of reversed cuticle, transpiration could be tenfold that of normal cuticle, though an equally efficient wax be present. We must, perhaps regretfully, conclude that a desiccation rate alone does not give a reliable criterion of the presence or absence of a wax layer; one must also consider the effect of the other shell components. Measurement of the permeability of the shell in both directions should, however, give diagnostic information.

Secondly, the air-sponge respiratory system of insect eggs (Wigglesworth & Beament, 1950) may materially affect suction force in a laminar system. A complete air layer in between two membranes limits water transfer to diffusion, and is known, both in models and (for example) in the dipteran puparium, to produce a most impermeable type of membrane system. It prevents the suction force of the dry outer membrane from affecting the inner one. The air sponge, even if filled with saturated air, will have a similar effect, but the proteinaceous pillars across it will act as a continuous liquid path, and so decrease its effect on transpiration.

Tracheal systems

It is early to do more than speculate on the implication of these ideas on water exchange in tracheal systems. So far as the main tracheal trunks are concerned, an identical exchange process to that of external cuticle is available to the insect; in the unwaxed tracheole one has usually considered capillary forces as the main source of activity tending to remove water from the tracheole end cell. (Such capillary forces may be much reduced by the presence of polar substances—octyl alcohol, for example—believed to be a component of cockroach grease as a solvent, which must, in the cockroach, and may, in other insects, contaminate the surface of the tracheolar water (Beament, 1951*b*, 1953; see also Wigglesworth, 1953). The process of removing water from the tracheole must involve the suction force of the tracheole lining, and desaturation of this lining by the end cell would readily provide the necessary mechanism. That water moves at such apparently great speed in the tracheole is suggested to be an illusion; true, if the water were only removed at the inner end of the tube, the meniscus would fall at an alarming rate, when one takes into account the viscosity of water in such a fine tube. But it is strongly suggested that there is no difference between the end of the tracheole and the rest of its length. Hence water is removed over the whole surface in contact with the liquid column, and there is no actual movement of the body of liquid down the tube. Were it possible to observe the withdrawal of water in slow motion, one imagines the liquid column to become hollow, giving the appearance of a falling meniscus, while there yet remained a wall of water against the membrane absorbing it. The energy requirement of this system is an increase in surface energy of the water, as the meniscus is expanded, and polar material would reduce this greatly.

Wax secretion

Among the problems outlined in this paper we have mentioned the difficulty of imagining the secretion of wax layers in the presence of a water-flow system, as a means of arresting water uptake. The suggestions made here minimize the need for such a hypothesis. Nevertheless, following the report of solvents in the cockroach cuticular grease, and their maintained secretion to offset evaporating solvent, we should consider the further possibility of such solvents in changing the permeability of cuticular wax, and thus providing the insect with an additional mechanism with which it may regulate the permeability of its cuticle. Cockroach and other cuticles and membranes have been placed in an apparatus to measure water diffusion between two humidities (as opposed to one humidity and liquid water). The details of this apparatus and results obtained with it will appear elsewhere, but a preliminary account of information so far obtained is relevant to this discussion. Solvent vapours have been injected into the air on both sides of membranes, and permeabilities obtained; the membranes have been stored under vacuum for days, and then returned to the apparatus. It is most significant that these cuticles show no reliable change in permeability in either direction when octane, decane or octyl alcohol vapours are present on both sides, in addition to the normal humidities; there is, indeed, a suggestion, using octyl alcohol, that permeability is decreased, but this may be due to the formation of monolayers on the reverse side of the cuticle. Artificially produced membranes show increased impermeability under these circumstances; vacuum treatment to remove solvents does not alter the permeability of either natural or artificial cuticles. On the other hand, chloroform, ether, benzene and acetone vapours all lead to an increased permeability, irreversible on storage in vacuum, and presumably due to permanent disorientation of the wax layer.

It seems striking that the particular solvents believed to be in cockroach grease could not affect permeability; they may even act as a piston oil to obtain and maintain a tightly packed monolayer. Subject to further investigation, then, solvents do not represent a mechanism whereby water exchange could be controlled.

We have taken no account, in these discussions, of the cement which is believed to cover the wax layer of certain insect cuticles. If, as some workers believe, this material is a tanned protein, then it would materially change the distribution of forces acting in the outer layers of the cuticle, and cause a considerable modification of the ideas expressed here. But the behaviour of *Rhodnius* cuticle and cast skin does not lead us to believe that the cement, in this animal at least, has any great effect on observed asymmetry. Obviously some considerable investigation into the composition of this material is of prime importance to our further understanding of the cuticle. Should the cement prove to be impregnated with waxy material, then this would remove its suction activity, while in no way interfering with its apparent usefulness as a mechanical protection to the underlying wax layer.

V. CONCLUSION

No suggestion is made that the many water-exchange problems in insects, some of which are reviewed above, are due entirely to inert physico-chemical processes. The evidence suggests that these processes are very much under the active control of living cells, which act by changing the gradients in their overlying membranes. The significance of wax layers is obviously much greater than the simple idea that they make an insect or egg 'waterproof and impermeable'. Epigrammatically, they have also apparently made water exchange and active secretion a much more available process to the terrestrial arthropod, and it is surprising that so few examples of water uptake, from vapour or liquid phase, are known to us.

I am particularly grateful to Dr T. O. Browning, and to Messrs M. W. Holdgate, R. Laughlin and M. Locke, for permission to include details of their unpublished work. Prof. V. B. Wigglesworth, F.R.S., Drs A. D. Lees, J. A. Ramsay, and others of the Department of Zoology, Cambridge, have been good enough to read and criticize the manuscript.

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THE EVIDENCE FOR ACTIVE TRANSPORT OF MONOSACCHARIDES ACROSS THE RED CELL MEMBRANE

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In the passage of the blood sugar between the human red cell and the plasma, certain complicating peculiarities have been apparent from the earliest investigations. Among mammalian erythrocytes, those of the primates appear to be unique in showing an appreciable degree of permeability to the hexoses. Moreover, even these cells fail to haemolyse appreciably when suspended in pure isosmotic glucose solutions, so that the entrance of the glucose appears to be limited in some manner. This is not evident, however, in the normal distribution of the human blood sugar, which appears to be uniform throughout the water of the cells and plasma (Kozawa, 1914; Ege & Hansen, 1927). Also, Klinghoffer showed in 1935 that there was rapid equilibration of glucose added in small amounts to that already present in the blood. Ege & Hansen concluded that the totality of information on glucose distribution in the blood was 'impossible to explain' in keeping with the natural assumption of the sugar's free solution in the two water phases.

Klinghoffer's investigations of the apparent paradox revealed that ready penetration of the sugar occurred only if the glucose concentration did not exceed about 2%. At higher concentrations, an extracellular excess was maintained almost indefinitely; and this unbalance was of sufficient degree to account for the failure of haemolysis to appear in isosmotic solutions. Bang & Ørskov (1937) measured this divergence from simple diffusion behaviour by showing, in a few experiments with varying glucose concentration in the neighbourhood of M/20, that the conventional red cell 'permeability constant' was approximately inversely proportional to the glucose concentration. Guensberg (1947) greatly extended this observation, finding that the variation of the 'constant', inversely with the glucose concentration, is over a range of at least a thousandfold.

Such behaviour implies some limitation on the absolute rate at which the glucose can move into the red cell; one suggestion is that the process requires participation of some ingredient of the barrier through which the sugar must pass to enter the cell interior. In recent years, much additional evidence has appeared in support of this view. Over the period 1946–52, while at the University of Vermont, I have frequently returned to this problem, and would like now to summarize the lines of evidence which indicate that the monosaccharides, in passing through the human red cell surface in either direction, temporarily combine with a 'carrier' molecule which is confined to that membrane or cortex layer. This evidence is in general along three lines:

(1) the kinetics of the sugar movements;
(2) the mutual interference with the movements in mixtures of sugars;
(3) the action of inhibitory substances.

In my own work, each of these lines was studied almost entirely by means of a single basic method, that of Ørskov (1935). This involves photometric recording of light transmittance through a very dilute suspension of red cells, as a means of following osmotic volume changes reflecting the movements of water across the cell surfaces. The general procedures and the operation of the recording system have been described elsewhere (LeFevre, 1948; LeFevre & Davies, 1951). The records show, as a function of time, the changes in direct transmittance which occur in response to various osmotically significant alterations in the composition of the medium (which consists of a buffered balanced salt solution to which the test substances are added). Since the suspension volume is at least 200 times as large as the total cell volume in these dilute suspensions, the concentrations in the medium are not appreciably altered by the cellular events, and may be treated as constant. Also, since the passage of water across the cell membranes, under a diffusion gradient, is much more rapid than the movements of the sugars with which we are concerned, it is legitimate to consider the osmotic pressure within the cells as identical with that of the medium at all times; the relatively slow volume changes recorded are then taken as a measure of the passage of glucose across the cell surface. Excellent linearity is found between the recorded quantity and the haematocrit or the calculated cell volumes in saline media of varying tonicity. When the sugars are present, small empirical corrections (LeFevre & LeFevre, 1952) must be taken into account if precise estimation of the cell-volume changes is to be attempted; but for any but the most critically quantitative work, direct inspection of the records is satisfactory for general analysis of the train of events. By arrangement of a suitable sequence of sudden alterations of the total osmotic pressure and the concentration of the penetrant in the medium, one can follow not only the entry of the substance into the cell, but also its subsequent exit.

The anomalous behaviour of glucose is apparent in the simplest series of this sort which was first attempted, in which glucose was simply added at various concentrations to a suspension of washed cells previously glucose-free. The pattern of the volume changes recorded in such an experiment is shown in Fig. 1. Certain clear deviations from the predictions of simple diffusion, as expressed in Fick's law, are immediately apparent. Approach to the equilibrium state is decidedly the more delayed, the more glucose is added; in fact, the initial rate of swelling actually decreases as the concentration of sugar is increased. At still higher concentrations, the rate of swelling diminishes markedly after the first few minutes, and drops to a nearly imperceptible rate, while the cells are still much too small for an even distribution of glucose to have been effected (for records see LeFevre, 1948). This latter special complication will be taken up later; the point of special interest in the pattern shown is that the apparent uptake of the sugar is not proportional to the gradient, but is limited to a maximum rate dictated by some other factor necessary for the translocation of the sugar.

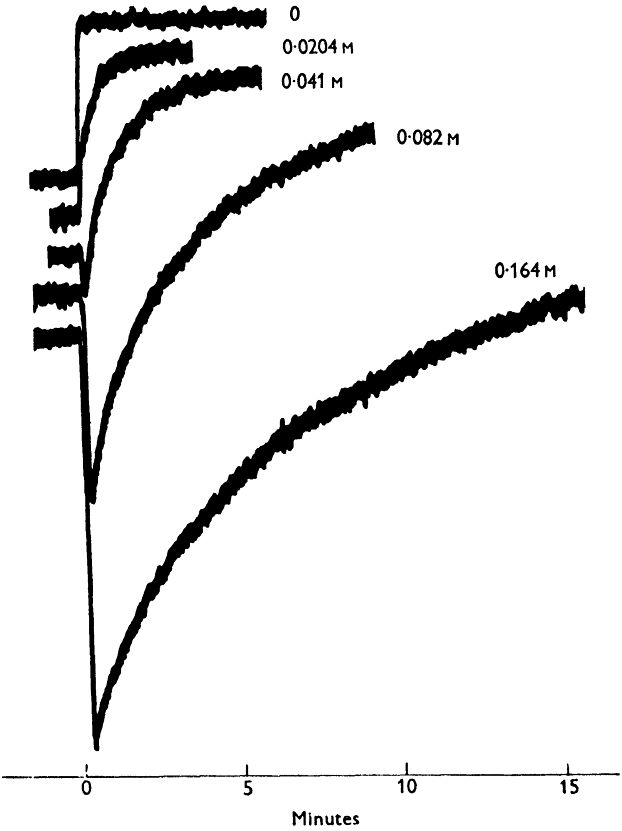

Fig. 1. Kinetics of swelling in glucose-saline mixtures. At zero time, 1 ml. of saline medium, containing glucose at 11 times final concentration shown, was added to 10 ml. of cell suspension (⅕ vol. %) in saline medium. (Medium here was only 0·6 × isotonic, so as to render rate differences more distinct.) 38° C. Immediate deflexion at zero time is resultant of dilution of suspension (upward deflexion) and cell-volume change (shrinkage downward); subsequent upward deflexion records cell swelling with uptake of sugar and water.

Such series of tests were run with all the hexoses and pentoses readily available: D-dextrose, D-laevulose, D-mannose, L-sorbose, D-galactose, L-arabinose and D-xylose. Among these, a clear dichotomy was apparent: all the aldoses (dextrose, mannose, galactose, and the two pentoses) behaved as just described; Fig. 2*b* shows, for instance, the behaviour of galactose. The two ketoses, laevulose and sorbose, as in Fig. 2*a*, on the other hand, seemed to obey reasonably well the predictions of Fick's law, and there was no reason to suppose any limiting factor other than the passive permeability of the cell membrane and the existing gradient for the sugar.

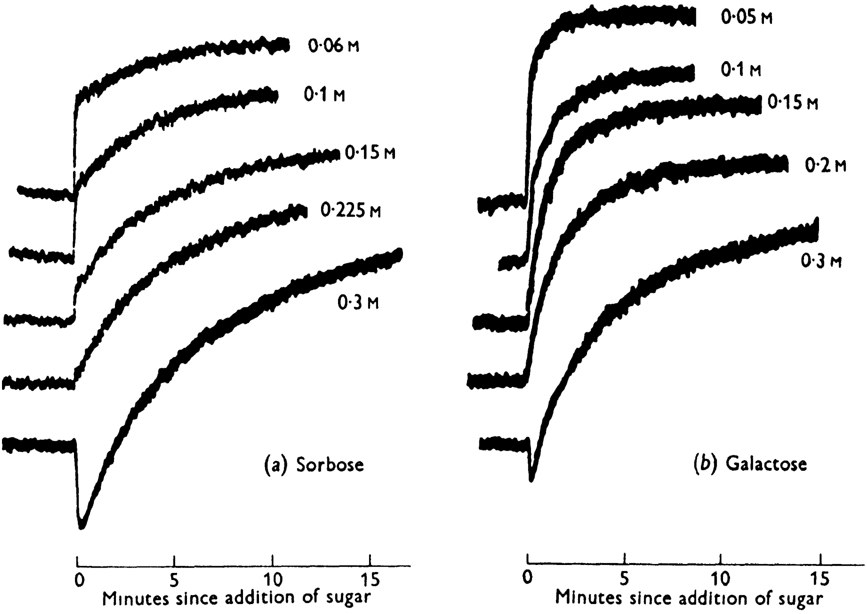

Fig. 2. Kinetics of swelling in sugar-saline mixtures. At zero time, 2 ml. saline medium, containing sugar at 6 times final concentration shown, was added to 10 ml. cell suspension (⅓ vol. %) in saline medium. 37° C. Deflexions interpreted as in Fig. 1.

This dissimilarity in behaviour of the aldoses and ketoses explains the discrepancy between the data of Kozawa (1914) and those of Wilbrandt (1938), with respect to the comparative rates of penetration of these sugars into the red cell. Kozawa, who worked with approximately ⅔-isosmotic solutions at room temperature, found (by haematocrit and direct chemical analytic methods) the following sequence, from fastest to slowest:

arabinose, xylose > galactose, mannose, sorbose > dextrose > laevulose;

while Wilbrandt found, with much lower concentrations of the sugars (and at body temperature), with an optical method:

xylose, arabinose > mannose > galactose > dextrose > sorbose ≫ laevulose.

The contrast in the pattern of dextrose and sorbose penetration as a function of concentration immediately accounts for the major disagreement between these two series. The other minor discrepancies are also attributable to the lesser differences between the sugars in this respect, in view of the differing concentrations at which the two investigators were working.

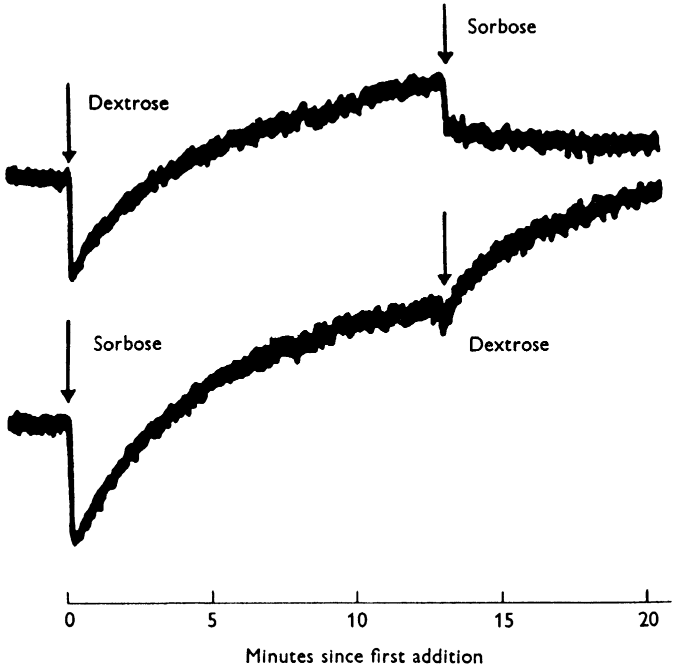

Fig. 3. Unilateral inhibition of uptake between sugars. At zero time, 1 ml. of saline medium, with sugar indicated at 1·8 M, was added to 10 ml. of cell suspension (⅓ vol. %) in medium; about 13 min. later, at time marked, a second 1 ml. was added, with sugar indicated at same concentration. Final concentration of each sugar was thus 0·15 M, or half-isosmotic. 37° C. Deflexions interpreted as in Fig. 1.

The obvious hypothesis to be derived from these observations was that the aldoses penetrate by a process involving participation of a cell component, while the ketoses penetrate simply by passive diffusion. This interpretation did not survive further experimentation concerned with the influence of the presence of one sugar on the rate of penetration of another. If, as seemed likely, all the aldoses shared a common transport system, there should be mutual interference with their entry when two or more aldoses are mixed; whereas the rate of entry of a ketose into the cells should be unaffected by the presence of other sugars of either type. This did not prove to be the true situation; instead, *all* the sugars appeared to be involved in a common reaction, so that in mixtures of any two the rate of swelling was always less than would be predicted on the basis of addition of the separate entries of each sugar. This was as true when ketoses were involved as with the aldoses. (For records, see LeFevre & Davies, 1951.)

Further light was shed on the situation by the procedure of adding the sugars serially rather than simultaneously, awaiting equilibration of the cells with the first before adding the second. In such experiments, the effect of the presence of the first sugar on the rate of entry of the second could be readily estimated in a quasi-quantitative manner. An example is provided in Fig. 3, which shows the characteristic situation between any aldose and either ketose. In the mixture of sorbose and glucose, each at 0·15 M, the entry of the sorbose is essentially completely prevented; whereas, prior addition of the sorbose had no effect on the later uptake of glucose, other than that attributable simply to its osmotic pressure. Similar relations were demonstrable between the two ketoses, and between any pair of the aldoses, except that the inhibitions were not always so overwhelmingly unilateral nor so absolute. Depending on the particular pair of sugars involved, the effect varied all the way from no detectable influence to apparently complete inhibition. The results of this entire series of experiments are summarized in Table 1, which indicates the relative effectiveness of each of the seven monosaccharides tested against each of the others. From this information, slightly modified by secondary factors discussed elsewhere (LeFevre & Davies, 1951), the avidity of the several sugars in attachment to the carrier molecule was considered to decrease in the order in which they are listed in Table 1, with the two pentoses being indistinguishable. The largest gaps appear to be between the aldoses and the ketoses, and between the two ketoses. Note that these relative affinities for the carrier do not define the relative rates of penetration, although such correlation improves as the sugar concentration is lowered.

Table 1. *Mutual inhibition in uptake of sugars*

In presence of	Inhibition of uptake of						
	Dext.	Mann.	Gal.	Xyl.	Arab.	Sorb.	Laev.
Dextrose	—	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + + +
Mannose	+ + +	—	+ + +	+ +	+ +	+ + + +	+ + + +
Galactose	+ +	+ +	—	+	+ +	+ + +	+ + + +
Xylose	+ +	+ +	+ +	—	+	+ +	+ + +
Arabinose	+ +	+ +	+ +	+	—	+ +	+ + +
Sorbose	o	o	o	o	o	—	+ + +
Laevulose	o	o	o	o	o	o	—

o No, or doubtful, effect.
\+ Just noticeable inhibition.
\+ + Moderate inhibition.
\+ + + Very marked inhibition.
\+ + + + Essentially complete block of uptake.

Another procedural variation, verifying the interpretation placed on these experiments, involved the simultaneous setting up of equal and opposite gradients for two sugars in a mixture. This was effected by equilibration with one sugar in a somewhat hypotonic saline medium, and subsequent addition of the second sugar together with a quantity of concentrated saline calculated to reduce the cell volume to the point that the outward gradient for the first sugar momentarily exactly equalled the inward gradient for the second. Thus the immediate cell volume assumed would correspond to the final equilibrium volume, and any intervening changes in volume reflect the net gain or loss as the two sugars move in opposite directions. Fig. 4 shows the behaviour of the cells in one such experiment; in this instance, opposing gradients for sorbose and dextrose are set up in each of the two possible arrangements. In both cases, the equilibration of glucose proceeded without impediment, while the sorbose movement was reduced to a small fraction of its uninhibited rate. The accessory records in Fig. 4 show the uncomplicated exit of the original sugar in the presence

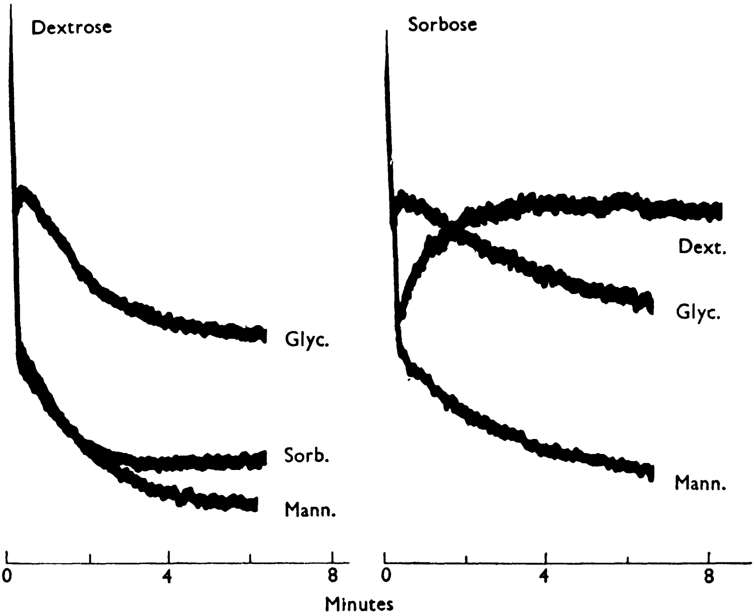

Fig. 4. Unilateral inhibition between sugars of movement in opposing gradient. Just prior to zero time, 10 ml. of cell suspension (½ vol. %) in 0·7 × isotonic saline medium had been equilibrated with either sorbose or dextrose (as labelled) at 0·262 × isosmotic. At zero time, 2 ml. was added, containing either glycerol, mannitol, sorbose, or dextrose, as labelled, at 1·5 × isosmotic, in saline medium at 4·0 × isotonic. This was calculated to reduce the cell-water volume from 1·43 to 0·80 × the 'normal', and thus set up an outward gradient for the original sugar of 0·25 isosmotic units, just equalling the inward gradient for the second non-electrolyte. 38° C. Deflexions interpreted as in Fig. 1. For significance of records see text.

of corresponding concentrations of mannitol (non-penetrating) and glycerol (penetrating much more rapidly than the sugars). Comparison with these control records makes it clear that in the one case the intracellular dextrose moves outward with almost complete exclusion of the sorbose; while, in the other instance, the intracellular sorbose cannot escape, so that after entry of the dextrose the cell volume remains near the maximum level attained at the beginning of the glycerol record (the record closely resembles the mirror image of that for glucose exit in the presence of mannitol). With either situation, the sorbose movement is reflected by only a very slow drift of the record back toward the final equilibrium level.

These indications of competition among all of the sugars tested led to early abandonment of the notion that only the aldoses shared the carrier system. Additional indication that the ketoses were similarly involved was found in the common sensitivity to inhibitory agents (discussed in a later section of this report); also, all showed a similar Q_{10} of the order of 3·0 (LeFevre & Davies, 1951). The fact that the ketoses failed to show the limitation on rate of uptake into the cells, which was observed with the aldoses, does not in itself militate against the hypothesis that the same carrier system is shared by both sorts of sugars. The appearance of a pattern resembling that of passive diffusion does not imply that necessarily no limiting reaction with the cell surface is involved in the movement of the ketoses into the cells, but might reflect simply a difference in relative velocity constants as compared with the case of the aldoses.

This is immediately apparent in considering the properties of the simplest model of the 'carrier system' that might be proposed. In the absence of any preliminary demonstration of the degree of complexity that might appear in (1) the formation of the sugar-carrier complex, (2) the movement or reorientation of the complex, or (3) the uncoupling of the sugar from the carrier, the least involved situation was first assumed. Steps (1) and (3) may be treated grossly in terms of only the net ingredients, so that any enzymic participation, or rate-limiting factors arising from step (2), are reflected only in the overall velocity constants; the following diagrammatic presentation emerges as representing the minimal essentials:

Outside Cell surface Inside

$$P + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A - P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A + P$$

C_s = conc. of P A_s = amount of complex $A - P$ S = amount of P

A = total amount of carrier S/V = conc. of P

$A - A_s$ = amount of uncombined carrier in which V is the cell-water volume, and k_1, k_2, k_3 and k_4 are the velocity constants for the several steps as labelled; the equilibrium constant for the reaction at the outer surface, K_1, is then equal to k_2/k_1, and similarly $K_2 = k_3/k_4$ for the interior reaction. Mass action law would then give the relations

$$\frac{dS}{dt} = k_3 A_s - k_4 (A - A_s) \frac{S}{V}, \tag{1}$$

and

$$\frac{dA_s}{dt} = k_1 C_s (A - A_s) - k_2 A_s - \frac{dS}{dt}. \tag{2}$$

No explicit solution of these equations to express S in terms of t appears to be possible; however, with glucose and the other aldoses, the observations noted above allow special restrictions on the system which simplify these relations. In the early stages of the process, while S is still a negligible factor, the rate of entry is essentially $k_3 A_s$, i.e. it is proportional to the amount of sugar-carrier complex. The finding that the process is limited so that no increase in initial rate occurs with increased concentration indicates therefore that the amount of this complex, A_s, remains nearly constant in the face of variation in C_s over the experimental range. (In Fig. 1, the initial slopes are approximately inversely proportional to total osmotic pressure.) This constancy of A_s indicates that the velocity constant k_3 is the factor limiting the overall transfer rate; that the reactions at the outer interface must be significantly faster than at the inner interface, so that A_s is nearly in equilibrium with the sugar in the external medium. Furthermore, k_1 must be considerably larger than k_2, since K_1 is evidently small compared to the lowest C_s at which the rate clearly ceases to increase with C_s.

The sequence of the sugars with respect to their competitive prowess in utilizing the carrier system, discussed above, is interpretable in the same terms. It presumably reflects the order of increasing K_1, the dissociation constant of the sugar-carrier complexes. The range of C_s in which the experiments of the type represented by Fig. 2 were carried out (about 0·05–0·3 M) defines a range of magnitude of K_1 apparently exceeding that of the aldoses, but not that of the ketoses. More precise calculation of this constant for the various sugars will be considered later from an entirely different experimental approach.

Assumption that K_1 is negligible compared to C_s seems then to be justified for the aldoses in the experimental range of C_s, at least in the case of glucose, the natural blood sugar in which we have the most interest. This, together with the conclusion that the outer reactions are near equilibrium by reason of the lower order of velocity constants at the interior, permits explicit solution of the equations. Also, since we are dealing experimentally with osmotic volume changes, we may treat C_s and S/V more properly as thermodynamic activities than as concentrations, and in these terms it is impossible for K_1 and K_2 to be unequal. With these simplifications, the earlier equations may be reduced to

$$\frac{dS}{dt}=Ak_3\left[\frac{C_iV_i-SC_m/C_s}{C_iV_i+S}\right], \tag{3}$$

in which V_i is the volume of the cell water at isotonicity (C_i), and C_m is the concentration of the non-penetrating components (salts) in the medium (all concentrations being expressed in osmotic terms). This may be integrated directly to give the relation of S and t; but since we are dealing with volume records, and since $S=V(C_m+C_s)-C_iV_i$, it is convenient to express the relation in terms of V:

$$\frac{dV}{dt}=Ak_3\left[\frac{C_iV_i-C_mV}{C_sV(C_m+C_s)}\right], \tag{4}$$

which may be integrated to give

$$t=\frac{C_s(C_m+C_s)}{Ak_3C_m}\left[V_0-V+\frac{C_iV_i}{C_m}\ln\frac{C_iV_i-C_mV_0}{C_iV_i-C_mV}\right], \tag{5}$$

in which V_0 is the cell-water volume when $t=0$.

This equation predicts that, *in any given mixture*, the course of volume changes will follow the pattern dictated by the laws of passive diffusion; but that, in the comparison of rates in *different* situations, the pattern will be entirely different from that derived from Fick's law.

The general applicability of this system to all situations with respect to glucose movements across the red cell membrane, in either direction, was tested in a wide variety of experiments involving as many contrasting situations as could be arranged. Usually, several factors were held constant while another was varied several times; for example, the initial cell volume, the initial glucose gradient, the initial cell glucose level, the total glucose transferred, the glucose level of the medium, or its total osmotic pressure. A number of examples of the results of such experiments, involving both outward and inward movements, have been illustrated elsewhere (LeFevre & LeFevre, 1952); space limitations here allow only one example, in Fig. 5. The match with the predictions from equation (5), which are shown for comparison, is evident, and was equally good for all circumstances tested, provided the extracellular glucose concentrations (C_s) were not allowed to exceed about 70% of isosmotic.

The significance of the rate equation (3) above, which gives this fit with experiment, is much more apparent after conversion to the following forms:

$$\frac{dS}{dt}=Ak_3\frac{C_s-S/V}{C_s}, \tag{6}$$

or

$$\frac{dS}{dt}=Ak_3\left(1-\frac{S/V}{C_s}\right). \tag{7}$$

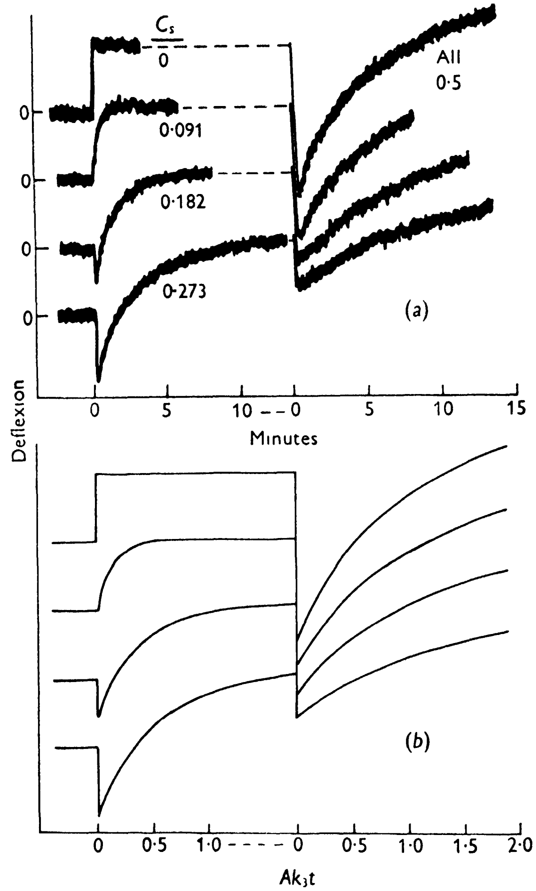

Fig. 5. Glucose entry in two stages, with fixed final C_s. (*a*) At zero time, to 10 ml. of cell suspension (⅓ vol. %), 1 ml. was added containing glucose at 11 × the concentration labelled; after equilibration, at second 'zero' time, an additional 1 ml. was added, containing glucose sufficient to bring the final concentration in each case to 0·5 × isosmotic, as labelled. All solutions contained salt mixture at 0·6 × isotonic. 37·5° C. (*b*) Pattern for same experiment, on basis of the hypothetical carrier system; scale for deflexions is matched approximately to fit lowest record in (*a*).

Equation (6) calls attention to the fact that the rate is at any instant *directly proportional to the existing gradient*, at a given extracellular concentration, but that with a given gradient it is *inversely proportional to the* *extracellular concentration.* The rearrangement in equation (7) combines these variables into a single term, i.e. the ratio of the intracellular glucose level to the extracellular; *the rate of transfer is proportional to the difference from unity in this ratio.* Thus the *inward* rate (positive dS/dt) can never exceed Ak_3, since the ratio cannot fall below zero; but the *outward* rate is not so restricted (the negative value of dS/dt will exceed Ak_3 whenever S/V is more than twice C_s).

Wilbrandt & Rosenberg (1950) found that the rate increased with increasing concentration on the upper end of the gradient only up to a certain maximum; and that the rate decreased with increasing concentration on the lower end of the gradient, but to a much greater degree than predicted by Fick's law. It is interesting to note that this is exactly what would occur according to the system developed above, if these experiments were performed by varying the external-sugar concentration, holding fixed the cell-sugar level. Table 2 illustrates how the carrier system would produce the results reported by Wilbrandt & Rosenberg, if this procedure were used.

Table 2. *Contrast of carrier and diffusion systems: effect of varying sugar concentration*

	C_s	Relative dS/dt by	
		Fick's law	Equation (7)
Entry: $S/V=0\cdot 1$, upper end of gradient varied	0·2	100—reference level	
	0·3	200	133
	0·4	300	150
	0·5	400	160
	0·6	500	167
Exit: $S/V=0\cdot 6$, lower end of gradient varied	0·1	−500	−1000
	0·2	−400	−400
	0·3	−300	−200
	0·4	−200	−100
	0·5	−100	−40

On the other hand, if the reverse procedure were followed, so that the cell-sugar concentration became the experimental variable, with a fixed level in the medium, then Fick's law and equation (7) would be indistinguishable, and the peculiarities seen by Wilbrandt & Rosenberg should not appear. Since these experiments have not been fully described, it is uncertain whether they offer additional support for the scheme developed here, or invalidate it.

The simple system definitely breaks down at sugar concentrations approaching isosmotic, which are of course far above the physiological norm. The transfer of glucose slows down markedly after the first few minutes, and may come essentially to a standstill while there is still a considerable gradient across the cell surface. Several possible explanations of this have been previously discussed (LeFevre & LeFevre, 1952). It was possible to reject on experimental grounds the suggestions of loss of major cell constituents, or of 'fixation' of the cells so as to preclude osmotic volume changes. The most likely interpretation consistent with the facts seems to be that the high glucose concentrations block the carrier reactions themselves. Wilbrandt & Rosenberg (1950) have in fact taken this view in a wider sense, claiming that the entire pattern of glucose movements suggests a case of enzyme inhibition by an excess of substrate. Although it does not appear from the experiments described above that this factor is involved appreciably in the operation of the system at reasonable sugar concentrations, it may well be the basis of its failure to function when C_s becomes excessive. In interpreting this type of inhibition as observed with DNAase activity, Cavalieri & Hatch (1953) point out that a molecule of water is involved in the cleavage of the sugar-phosphate bond, and suggest that the substrate may compete with water for a site on the enzyme. This hypothesis could equally well be applied in the present instance.

That the complex formed in the membrane is in fact a sugar phosphate has in no way been indicated directly by the work reviewed here; but involvement of some enzymic factor is implied. The operation of inhibitors has been suggestive; inhibition of the uptake of glucose into red cells was effected by very small concentrations of Hg^{++}, Hg_2^{++}, or *p*-chloromercuribenzoate (LeFevre, 1947, 1948), and by chloropicrin, bromacetophenone, allyl mustard oil, or gold (Wilbrandt, 1950). (Iodine is also an effective inhibitor, but only at concentrations which also lead to an obvious discoloration of the haemoglobin.) The efficacy of this group of substances suggests that some part of the transport process involves sulphydryl groups; if so, these groups are evidently of the not easily available type characterized by Barron & Singer (1945), since there appears to be no inhibition at all by Cu^{++}, alloxan, mapharsen, iodoacetate or arsenite (LeFevre, 1948).

Use of another class of inhibitors has more recently been particularly fruitful in the analysis of the carrier mechanism; I refer to the glucoside phlorizin (generally considered to be rather specifically active against phosphorylation transfer systems), and its aglucon, phloretin (*β*-(*p*-hydroxyphenyl) 2, 4, 6-trihydroxypropiophenone). Either of these agents acts as a block to the transfer of the monosaccharides across the human red cell surface; but as Wilbrandt (1950) has shown, the simpler molecule, phloretin, is many times more effective than its glucoside phlorizin. Wilbrandt expressed the conviction that these agents act on the process by which the sugar *emerges* from the membrane (whether this be on the inside or the outside) rather than on the step of entry into the membrane. He could show inhibition of glucose exit from the cell without any disturbance of its entry, when (by reason of slow penetration) the agent was more concentrated in the external medium than within the cell. Wilbrandt suggests that phosphorylation by hexokinase is concerned in the initial step, and dephosphorylation by a phosphatase in the second step, and that it is this latter step which is sensitive to phlorizin and phloretin. In Wilbrandt's scheme, the system does not simply consist of a reversible set of reactions, but involves different operating units according to whether the sugar is entering or leaving the cell. With such a system it is difficult to account for the apparent failure of glucose ever to accumulate against a concentration gradient in these cells; whether a reasonable fit with the observed kinetics could be achieved with this system has not been considered.

Wilbrandt's published statements with regard to the peculiar action of phloretin in selective inhibition of the exit process have been so far only qualitative descriptions; a complete statement of procedure would be helpful, as without this it is impossible to determine whether the observations actually refute the simpler interpretation of the inhibition under the scheme offered here. My own experiments are entirely in accord with the hypothesis that the phloretin acts on the first reaction involved, by *direct competition* with the sugars for combination with the carrier molecule (or the limiting molecule involved in the chain leading to formation of the carrier complex). The following analysis, derived from this hypothesis, has in fact permitted rough calculation of the dissociation constants of some of the carrier complexes.

If the inhibitor acts at low concentrations by combining with the carrier in the same manner as do the sugars, it must have considerably higher affinity for the carrier (a much smaller K). Thus, when an extra ingredient of this type is added to the former system,

$$\frac{dS}{dt}=\frac{A(k_3 C_s/K_s-k_4 S/V)}{1+C_I/K_I+C_s/K_s}, \tag{8}$$

in which C_I and K_I are respectively the concentration and equilibrium constant for the inhibitor and K_s is the equilibrium constant for the sugar (equal to K_1 or K_2 of the original system). The ratio of the uninhibited rate (R_0) to the inhibited rate (R_I) is then given by the relation

$$\frac{R_0}{R_I}=1+\frac{K_s C_I}{K_I(K_s+C_s)}. \tag{9}$$

Thus, in a plot of this ratio against C_I, in a series of tests in which only C_I is varied, a straight line should be obtained, the slope of which is

$$K_I^{-1}(1+C_s/K_s)^{-1}.$$

This rectilinearity is observed experimentally, as shown in Fig. 6. The records of cell shrinkage during glucose exit, in a series of concentrations of phloretin, under an otherwise constant set of conditions, are given in Fig. 6*a*. From such records the relative initial rates of glucose loss may be estimated and compared as a function of the inhibitor concentration. Fig. 6*b* shows the data of this same experiment, plotted in the manner prescribed above; a similar set of data for inhibition by Hg^{++} is included for comparison, showing that with this agent the inhibition is clearly not of the competitive type.

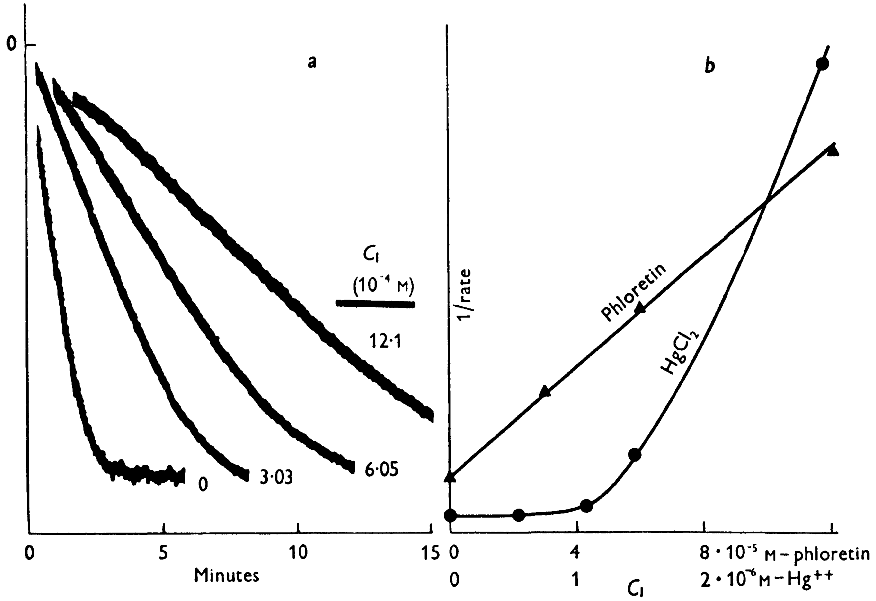

Fig. 6. Inhibition of glucose exit as a function of inhibitor concentration. (*a*) A 3 % cell suspension was equilibrated at 38° C. for 1 hr. with 0·5 × isosmotic dextrose in 0·7 × isotonic saline medium. Then, at zero time, 2 ml. of this was added to 10 ml. of the saline medium, containing phloretin so as to make the final concentration of the inhibitor as labelled in the figure. (*b*) The data of (*a*), and a similar experiment with $HgCl_2$ in place of the phloretin, plotted as suggested in the text.

More convincing evidence of the competitive nature of the phloretin inhibition is obtained from consideration of the effect of varying the sugar concentration, with a fixed inhibitor concentration. Equation (9) may be rearranged

$$\frac{R_I}{R_0 - R_I} = \frac{K_I}{C_I}\left(\frac{C_s}{K_s} + 1\right); \tag{10}$$

so that if $R_I(R_0 - R_I)^{-1}$ is plotted against C_s at a fixed C_I, it should yield a straight line with $-K_s$ as the *x*-intercept and K_I/C_I as the *y*-intercept. By this means, then, both K_I and K_s can be estimated. Such a graph, for inhibition of glucose exit by phloretin, is presented in Fig. 7; this experiment gives a glucose K_s of 0·009 M, and for phloretin a K_I of $4{\cdot}9 \times 10^{-6}$ M. Thus the inhibitor's 'affinity' for the carrier appears to be about 1800 times that of the sugar.

The useful measurements obtained by this approach are summarized in Table 3. The work was necessarily cut short soon after the initiation of this phase in August 1952, and it was impossible to gather a full complement of data for estimation of K_s of each of the sugars and to check the K_I for phloretin and phlorizin using each of the sugars as test penetrant. However, the legitimacy of the interpretation of the observed rectilinearity in the plotted relations is attested by the finding of reasonably similar values in the constants with different experimental procedures. It is especially to be noted that the dissociation constants for sugar and inhibitor are of similar magnitude in experiments with outward movement as with inward movement. Most reassuring perhaps is the finding of the same range of value for K_I with different sugars having quite different K_s.

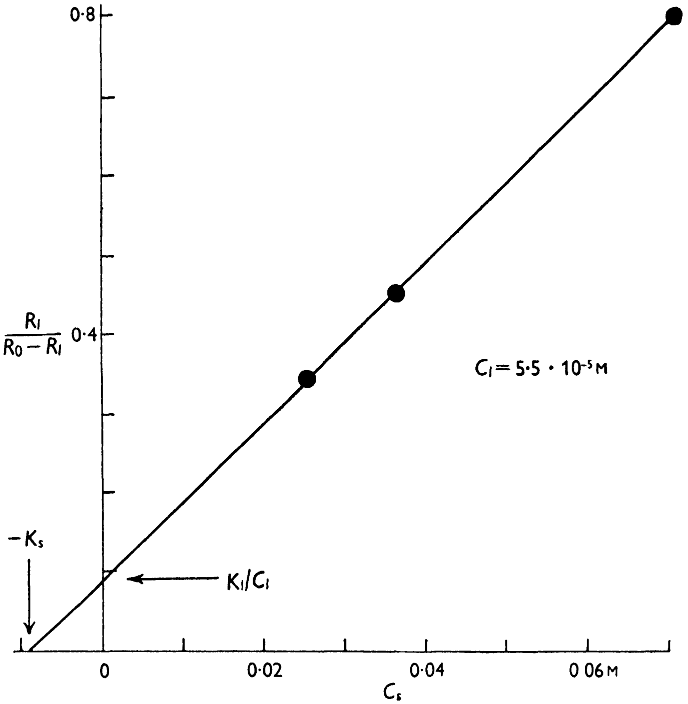

Fig. 7. Inhibition of glucose exit by phloretin as a function of glucose concentration. Procedure as with Fig. 6a, except that glucose was added in varying amounts to the final mixture; at each C_s, two runs were taken, one with and one without phloretin at $5{\cdot}5 \times 10^{-5}$ M. For rationale of system of plotting data, see text.

These results lend new support to the previously postulated interpretation of the differences in behaviour between the aldoses and ketoses, and of the pattern of competitive inhibition between the various sugars. Thus, the value found for glucose K was appreciably smaller than the C_s range useful in the experimental procedures; that for galactose was at the lower edge of this range, whereas the K for the two ketoses was in excess of the upper experimental limit for C_s. The practical limitations on C_s, in fact, made it impossible to ascertain the ketoses' equilibrium constants with any satisfactory degree of precision. In contrast to the situation with the aldoses, the concentration of the ketoses (C_s) did not affect appreciably the degree to which a given concentration of inhibitor would act. The plot of $R_I(R_0 - R_I)$ against C_s therefore gave for the ketoses a line of such low slope that the location of its x-intercept was a matter of enormous uncertainty. Nevertheless, the experiments with the ketoses gave a similar K_I for phloretin, and showed equally good rectilinearity in the plot of R^{-1} against C_I. All this is in complete accordance with the theoretical relation derived above, in the contrast of the situation $K_s \gg C_s$, with the situation $K_s \ll C_s$.

Table 3. *Estimation of carrier-complex dissociation constants*

Inhibition of	Sugar K_s	Phloretin K_I*	Phlorizin K_I†
Dextrose entry	$7{\cdot}5\times10^{-3}$ M	$4{\cdot}5\times10^{-6}$ M	—
Dextrose entry	10×10^{-3} M	—	—
Dextrose exit	9×10^{-3} M	$4{\cdot}9\times10^{-6}$ M	—
Dextrose exit	8×10^{-3} M	—	—
Dextrose exit	8×10^{-3} M	—	—
Dextrose exit	$7{\cdot}5\times10^{-3}$ M	—	$1{\cdot}45\times10^{-4}$ M
Galactose entry	$5{\cdot}0\times10^{-2}$ M	—	—
Galactose exit	$4{\cdot}4\times10^{-2}$ M	$4{\cdot}8\times10^{-6}$ M	—
Sorbose entry	1·3–2·0 M‡	—	$1{\cdot}27\times10^{-4}$ M
Sorbose exit	*ca.* 2 M‡	$4{\cdot}4\times10^{-6}$ M	—
Laevulose entry	*ca.* 2 M‡	$4{\cdot}4\times10^{-6}$ M	—

* Five values given represent complete series permitting plot as in Fig. 7; where no value is listed, phloretin K_I was taken as $4{\cdot}7\times10^{-6}$ M in calculation of K_s from plot as in Fig. 6*b*.

† Two values given are on basis of parallel tests with phloretin, taking K_I of latter as $4{\cdot}7\times10^{-6}$ M.

‡ Sorbose and laevulose K cannot be satisfactorily estimated in these experiments; see text for discussion.

In summary, then, the several lines of attack have all fitted into the schematic system illustrated above. This does not mean that the actual mechanism may not be considerably more complicated, with extra steps involving additional components, perhaps enzymic, which are not specifically included in the postulated system. It does indicate, however, that any such additional steps do not represent separate rate-limiting factors, so that for kinetic analysis they can be lumped together into the two overall reactions dealt with here.

Beyond the suggestiveness of the nature of the inhibitors found to be effective, there has not been any indication in this work of the nature of the carrier-complex or of the probable enzymic factors involved. Phosphorylation is of course the obvious suggestion; glucose-6-phosphate does not measurably penetrate the red cell, however, and it seems unlikely that the complex which is supposed to be confined to the surface layer would show this inability to enter that layer from the medium. Wilbrandt (1950) has described a possible form of hexose-metaphosphate which should be unionized and fairly fat-soluble, and has suggested that this could well be the complex involved; there does not seem to be any direct evidence of this at the moment.

Finally, it should perhaps be emphasized that, whatever the details of the mechanism may prove to be, there is no evidence that the red cell is equipped with a hexose 'pump' that can provide the energy for transporting sugar against a concentration gradient. The data merely indicate that there is a temporary complex formed between the sugars and a cell-surface component during the transfer (in a somewhat circumscribed manner); and this is apparently the basis for the peculiar ability of the primate erythrocyte to take up these substances.

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SECRETION AND TRANSPORT OF NON-ELECTROLYTES

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I. INTRODUCTION

Active transport of non-electrolytes has been observed mainly in connexion with nutrition and excretion as well as with osmoregulation (transport of water).

This report will concentrate on the transport activity of the epithelial cells in the intestine and the kidney as well as that of red cells. Special attention will be given to monosaccharides. There will be no discussion of active transport of either water or inorganic cations which are covered in other papers.

If, however, weak electrolytes, both acids and bases, were excluded in strict accordance with the title of the paper, the list of actively transported substances would become rather small.

This seems of some interest, because it provides a partial answer to the first question to be asked: *Which sort of substances are apt to be transported actively?*

Substances which appear to be actively transported include the common foodstuffs, sugar, amino-acids, possibly fats and a larger series of waste products as well as foreign substances.

Sugars and amino-acids are transported in the intestine as well as in the mammalian kidney, due to the particular mode of action of this excretory organ involving at first filtration so to speak of the entire 'milieu interne' and then reabsorption of those components that are essential to the organism.

The mode of working of the mammalian kidney has been elucidated first by the classical work of A. N. Richards and his group with micropuncture technique on single nephrons. Later, on the basis of the results so obtained, extensive work on the whole kidney by various groups, including those of H. W. Smith, Shannon, Pitts and others, has made available a large body of information. (A comprehensive survey has recently been given by H. W. Smith (1951).)

The function of the kidney consists of three elements: filtration in the glomeruli, reabsorption in the tubules and secretion in the tubules. Whereas filtration so far has been considered as passive, reabsorption may be and secretion always is an active process. Active reabsorption is assumed if the concentration ratio urine to plasma (U/P) falls (or may under certain conditions fall) below 1·0. Particularly this is the case with the so-called 'threshold substances' which appear in the urine only when a threshold concentration in plasma is surpassed. They include sugars, amino-acids, urea in the kidney of elasmobranchs, lactic acid, phosphate, sulphate, uric acid, ascorbic acid and possibly more substances that have not been investigated yet. With the apparent exception of uric acid, they are all substances which serve some special purposes in the body and may be called essential substances.

Table 1 *a* lists substances which are actively reabsorbed.

Secretion, with few exceptions, is restricted to organic electrolytes, both weak and strong acids and strong bases (Table 1 *b* and Fig. 1). They include the group of iodinated X-ray contrast substances which have been selected commercially, according to their ability to be concentrated in the urine, without knowledge of the mechanism involved, and have later been among the first cases to be recognized as secretion. Their iodine-free nuclei behave in the same way, showing that iodine as such is not essential for the reaction of the kidney tubule cell. There are, furthermore, several derivatives of hippuric acid which for a long time has been known as a product of 'detoxification'. Höber (1945) and later Sperber (1947) have pointed out that an important feature of many detoxification processes is the formation of rapidly excretable compounds. Furthermore, there is penicillin whose rapid excretion has long been one of the foremost problems in practical penicillin therapy, and caronamide which was introduced into therapy to

Table 1. *Actively transported substances in the kidney*

1 *a*. Absorption	1 *b*. Secretion
Glucose	Tetraethyl-ammonium
Xylose	Methyl-nicotinamide
Fructose	Phenol red
Galactose	Penicillin
	Ponceau R
β-Hydroxy butyric acid	Caronamide
Lactic acid	Hippurate
Uric acid	*m*-Amino-hippurate
Ascorbic acid	*p*-Amino-hippurate
	p-Acetyl-amino-hippurate
(Urea)	Hippuran
Glycine	*o*-Hydroxy-hippurate
Alanine	Diodrast
Glutamic acid	Iopax (Uroselectan)
Creatine	Neo-iopax
	2-Pyridine-1-acetic acid
Lysine	Skiodan
Arginine	
Histidine	
Leucine	
Isoleucine	

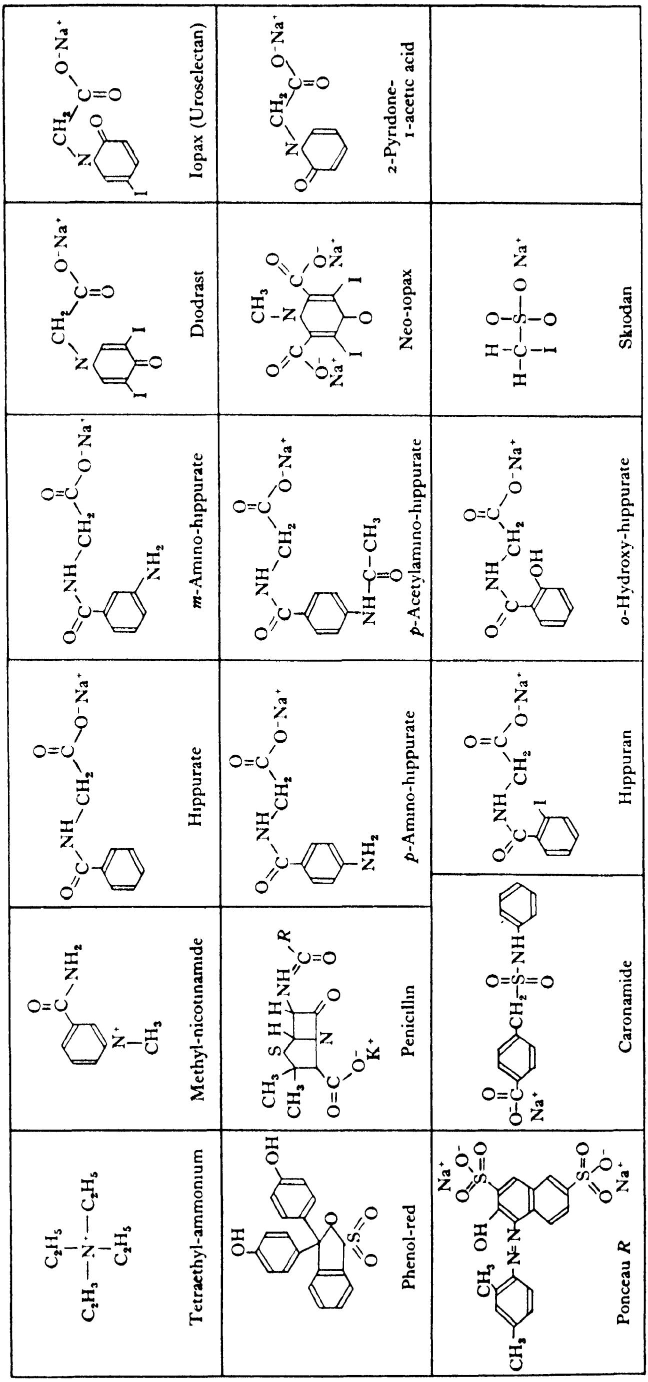

Fig. 1. Structures of molecules secreted through the kidney tubule.

compete with penicillin in the kidney. Phenol red was historically the first substance whose secretion was experimentally established. The sulphonic acid azo dyes studied by Höber & Briscoe-Woolley (1940) are strong acids. Finally, methyl-nicotinamide and tetraethyl-ammonium are quaternary ammonium bases.

In the intestine less information is available. Examples of active absorption are known mainly among the foodstuffs: sugars, amino-acids and possibly fats. The question of secretion in analogy to the kidney has as yet been very little investigated. It was found, however, that diodrast is actively secreted in the small intestine (Smith, 1951).

A survey of these actively transported substances shows that those reabsorbed are strongly hydrophilic, while those secreted quite generally have molecules possessing both hydrophobic non-polar and hydrophilic polar groups (some of the latter ionizing), indicating that this polar/non-polar type of molecular structure favours secretory transport. Höber (1940), in a study of a large number of sulphonic acid azo dyestuffs was able to show that, in the case of the kidney, as a rule only those dyes are secreted which are asymmetric with respect to the sulphonate group, possessing one or two groups on one ring but none on any other.

With respect to the permeability of the cell membrane this rule indicates that a high ability of penetration is not, as might be expected, a condition of secretory transport. On the contrary, we find in the list practically only one compound known to penetrate cell membranes easily, namely, urea, whose secretory transport, however, is a specialized case in the group of elasmobranchs. Several compounds related to hippuric acid and diodrast have been shown not to penetrate red cells (*m*-amino-hippurate, *p*-amino-hippurate, *p*-acetyl-amino-hippurate) or only slowly, not reaching equilibrium *in vivo* (*p*-hydroxy-hippurate, *o*-hydroxy-hippurate, cinnamoyl glycine, hippuran, diodrast, iopax). Amino-acids, quaternary ammonium compounds, and in most species the sugars, also penetrate either with extreme slowness or practically speaking not at all. This rule and its bearing for the transport mechanism will be referred to later.

II. CHARACTERISTIC FEATURES OF ACTIVE TRANSPORT

Before the mechanism of active transport is discussed, some remarks seem appropriate with respect to several features more or less common to those transports, in contradistinction to diffusion processes. From any theory advanced for the transport mechanism an adequate explanation of these features should be required.

(1) *Transport against the gradient of electrochemical potential*

These transports (which have also been termed 'uphill transports') are the only ones for which the epitheton 'active' is not disputable (cf. Rosenberg, 1948). They are opposite to the thermodynamic tendency and cannot occur spontaneously without energy-providing mechanisms.

Both kidney and intestine perform 'uphill transports'. In the kidney, as far as reabsorption is concerned, this is obvious for all substances with U/P ratios smaller than 1. They include the threshold substances as well as fructose, galactose and others.

For substances secreted in the tubules, uphill transport is indicated if the U/P ratio is larger than that for inulin (which is only concentrated by reabsorption of water in the tubules). This is true for all the substances listed in Table 1 *b*. Most of them have U/P ratios close to that of *p*-aminohippurate (which is about 5·3 times higher than that of inulin); some have lower ratios, down to 3. If no back-diffusion occurs, these values are minimal figures for the accumulation ratio in the secretory transport. They hold if the transport delivers the substances into the tubular urine before reabsorption of water begins. If the level of entrance into the tubule is lower than the level of the beginning of water reabsorption, the accumulation ratio must be higher to account for the final U/P ratio.

In the intestine, convincing evidence for 'uphill transport' has been furnished by Barany & Sperber (1939) with respect to the absorption of glucose. In this study elimination of the disturbing factor of water reabsorption was accomplished by the addition of sodium sulphate, which is poorly absorbed by the intestinal epithelium.

(2) *Non-linear rate-concentration relationship*

The rate of a diffusion process when following Fick's law is proportional to the slope of the concentration gradient. In the case of diffusion from finite to zero concentration (or in the case of the diffusion flux) it is proportional to the concentration: $dA/dc = \text{constant}$ ($A = \text{rate of transport}$, $c = \text{concentration}$).

For active transports this is not true. With rising concentrations dA/dc decreases. In many cases it was found finally to reach zero, indicating a maximum rate of transport, which cannot be further increased by raising the concentration. In kidney physiology this maximum rate has been designated as T_m.

For the absorption from the intestine dA/dc was studied with respect to sugars by Cori (1925), Verzàr (1935), Höber & Höber (1937), Barany & Sperber (1942), and by Vidal-Sivilla (1950), with respect to amino-acids by Höber & Höber (1937). The results show definitely that dA/dc is not constant. The shape of the rate-concentration curve was not studied in detail for amino-acids and could not be established very clearly for sugars due to considerable scattering of the data. It seems, however, doubtful whether a maximum rate is actually attained with high concentrations. In the range studied it was not reached. It has been assumed (Verzàr, 1935; Donhöffer, 1935) that absorption is composed of two fractions, an active process and passive diffusion. In this case a constant maximum rate of absorption could not be reached because the diffusion fraction would continue to increase with rising concentrations.

More data are available with respect to reabsorption and secretion in the kidney. As to reabsorption, non-linearity of the rate-concentration function is clearly shown by the existence of the 'threshold substances' mentioned above. (As 'threshold' critical levels of plasma concentrations are designated, above which excretion in the urine occurs, whereas with lower concentration reabsorption is complete.) Threshold substances include glucose, amino-acids, lactic acids, phosphate and sulphate.

Non-linearity, however, is not limited to the group of threshold substances. It has been observed in a large number of cases, both of reabsorption and of secretion. In many of them (but by no means in all) with high concentrations a maximum rate of transfer was reached.

Fig. 2 shows excretion and reabsorption of glycine (Pitts, 1943) in the dog as a function of the filtered amount per minute (filtrate volume per minute times plasma concentration). Both curves clearly show that with high values of the filtered amount a constant maximum rate of reabsorption is reached. This maximum rate is approached with different rapidity for different substances, e.g. more rapidly for sulphate than for glycine.

With respect to the form of the A/c curves and their differences reflected in the curves of Fig. 2 some remarks seem useful.

Since the filtered volume per minute (glomerular filtration rate) varies relatively little, the abscissa is approximately proportional to the substrate-plasma concentration and consequently to the concentration in the glomerular filtrate entering the tubule. Nevertheless, the curves designated 'reabsorption' (dots) should not be taken as showing the form of the A/c function. The amount reabsorbed per unit time, T, is an integrated value, the sum of the amounts reabsorbed at all levels of the tubules with decreasing values of c. It may be calculated for a given form of the A/c dependence. In Fig. 3 curves are shown which have been calculated on the (arbitrarily chosen) basis of the Michaelis-Menten equation

$$A = K\frac{c}{c+K_m}, \tag{1}$$

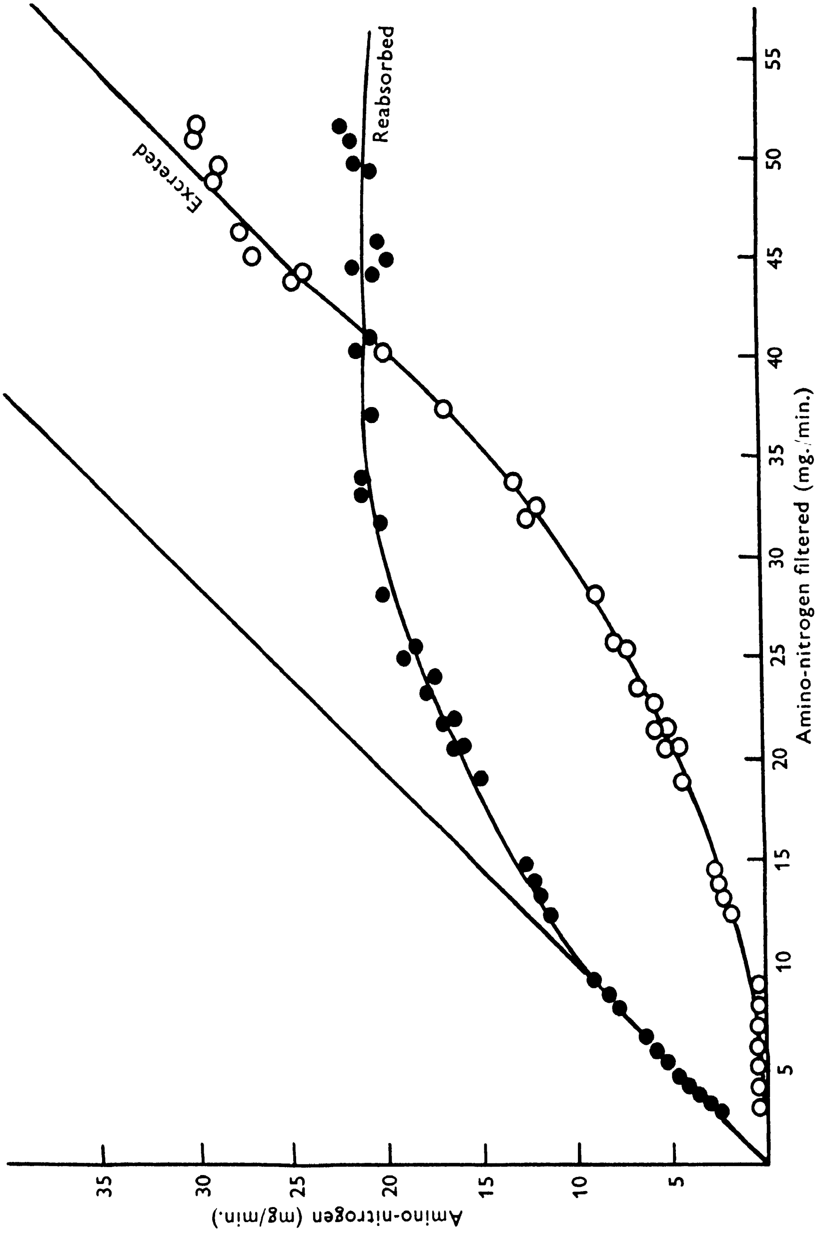

Fig. 2. Reabsorption of glycine in the kidney tubule of the dog. Data from Pitts (1943).

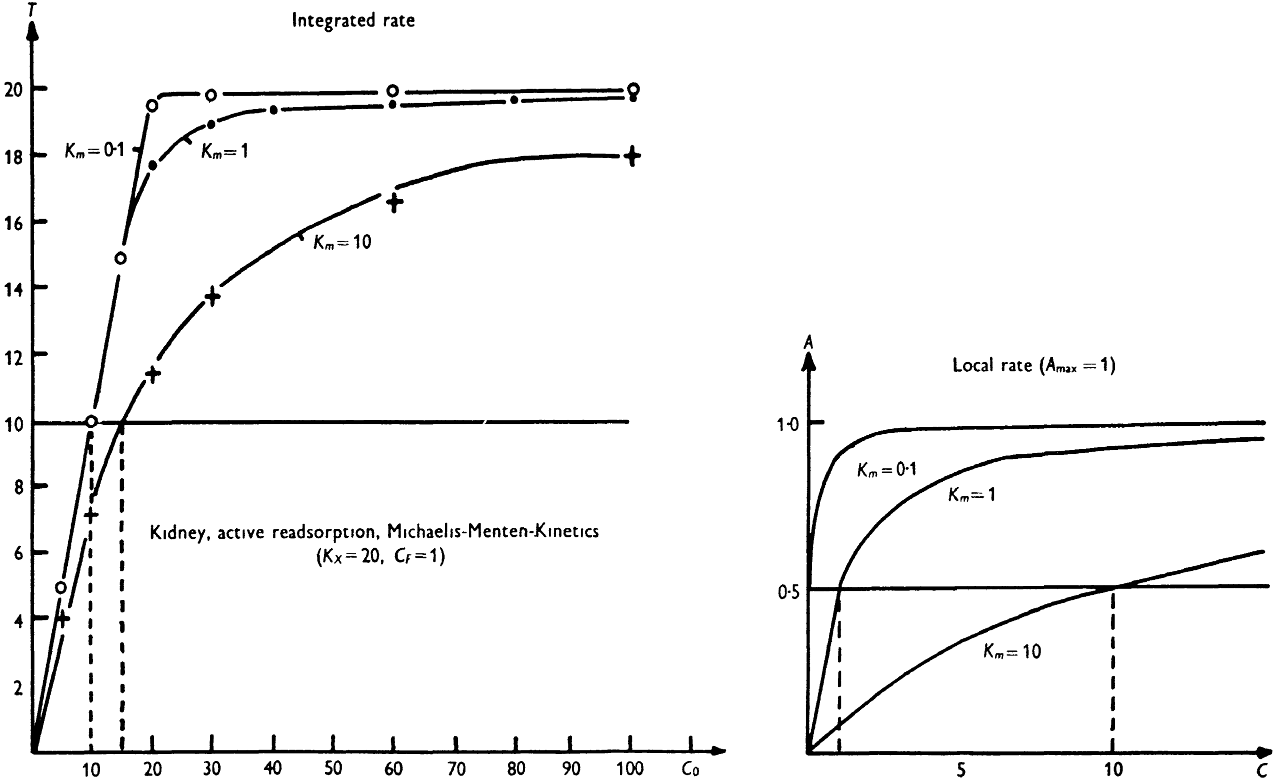

Fig. 3. Local and integrated rate of reabsorption calculated on the basis of a Michaelis-Menten relation between rate of reabsorption and concentration.

where A is the amount reabsorbed per unit length of the tubule and unit time, c the concentration, K_m the Michaelis-Menten constant and K a second constant (in an enzymic reaction indicating the enzyme concentration). T was obtained by integration over the entire tubule, using the relations

$$dT = A dx \tag{2}$$

and

$$T = C_F(C_0 - C), \tag{3}$$

where x is the distance from the beginning of the tubule, T the amount reabsorbed per unit time between the beginning of the tubule and the distance x, C_F the volume filtered per unit time (rate of glomerular filtration) and C_0 the concentration in the glomerular filtrate.

The integration yields the transcendental equation

$$Kx = T + C_F K_m \ln \frac{C_F C_0}{C_F C_0 - T}. \tag{4}$$

The curves in Fig. 2 were calculated for three values of K_m: 0·1, 1·0 and 10·0 (assuming a constant value of 20 for Kx).

They resemble those of Fig. 2 in the general shape. The difference mentioned between glucose and sulphate, under the assumptions used here, would appear to reflect differences in K_m.

It should be noted, however, that the T/C_0 curves are by no means identical with the A/C curves, as Fig. 3 shows. For instance, the concentrations for 50% maximum rate are $c = 0{\cdot}1$, 1 and 10 with respect to A, but $C_0 = 10$, 10 and 15·6 for T.

Thus from T/C_0 curves the function A/c cannot be evaluated directly.

The Michaelis-Menten relation was chosen arbitrarily. It is not likely that the true function is equally simple. It is very probable, however, that it will contain functionally homologous factors: a factor determining the maximal rate, homologous to K in the Michaelis expression (indicating the enzyme concentration) and another factor determining the rapidity of approach to the maximal rate with rising concentrations, homologous to K_m. They may be termed *extensive* and *intensive* factors.

As would be expected the values of T_m vary considerably. For the comparison of data obtained in different species it has become customary to reduce them to 1 m.^{2} of body surface. So calculated the following values of T_m in mmol./min. m.^{2} of body surface have been reported (taken from Smith (1951) and changed into the units adopted here):

Glucose man ♂	1·2	Leucine dog	0·64–0·71
Glucose man ♀	0·97	Sulphate dog	0·08–0·1
Glucose dog	0·67	Phosphate man	0·075
Glycine dog	1·29–2·29	Uric acid man	0·056
Valine dog	0·93	Ascorbic acid man	0·007

The values of T_m appear to reflect roughly the degree of importance of the substances as metabolites. The highest figures are those for glucose and for glycine.

It seems useful to refer one of these values to unit surface area of the absorbing tubule cells. The total number of nephrons in man are estimated at two millions, the length of the proximal tubule is given as 13 mm., the diameter of the lumen as about 20μ. This would give a total surface area of $1{\cdot}6 \times 10^4$ cm.^{2} ($= 1{\cdot}6$ m.^{2}!). T for 1·73 m.^{2} body surface (instead of 1 m.^{2} as listed above) is 373 mg. /min. = 2 mmol./min. (which per day amounts to a transported quantity of 480 g.). The amount reabsorbed per second and cm.^{2} surface area thus would be $0{\cdot}21 \times 10^{-8}$ mol. This value will be referred to later.

For secretion likewise constant maximal rates at high concentrations or at least non-linear relationship between rate and concentration are observed. Diodrast T_m values determined on man range from 36 to 52·8 mg./min. and 1·73 cm.^{2} body surface, the average being higher for males (49·9) than for females (42). T_m for *p*-amino-hippurate in the same units is 53 mg./min. In terms of moles these values of T_m (0·117 mmol. for diodrast and 0·273 mmol. for *p*-amino-hippurate) are considerably lower than for glucose. Phenol red shows a definite maximal rate not only in the glomerular kidney (T_m for man 35·8 mg. = 0·103 mmol./min. per 100 ml. glomerular filtrate, for the dog less), but also in the aglomerular kidney, where its demonstration by Marshall & Crane as early as 1924 was one of the first observations of this kind.

(3) *Competition*

If the maximal rate of transport is interpreted as due to a limited capacity of the transport mechanism, competition among substances transported by the same mechanism is to be expected. Several examples are known.

In the intestine Cori (1926) showed that the amount absorbed from a mixture of two sugars was less than the sum of the amounts absorbed from solutions of the single sugars in the same concentrations, in fact, it was only about equal to each of these amounts.

Again in the kidney more data are available. Several examples are known of transport substrates inhibiting the transport of others, both in reabsorption and in secretion.

With respect to reabsorption saturation of the transport mechanism by high concentrations of glucose blocks the reabsorption of xylose completely, that of fructose incompletely (Gammeltoft & Kjerulf-Jensen, 1943) or not at all (Levine & Huddlestun, 1947). Saturation with glycine, alanine or glutamic acid blocks the reabsorption of creatine (Pitts, 1943, 1944). Other pairs of substances with mutual inhibition of reabsorption are leucine and isoleucine, arginine and lysine (Beyer, Wright, Skeggs, Russo & Shaner, 1947). The reabsorption of amino-acids, however, was not affected by saturation with glucose.

Thus several groups of substances appear to be transported by separate mechanisms, members of one group competing with each other, but not with members of other groups. According to Beyer *et al.* (1946, 1947) and to Pitts three groups of amino-acids may be distinguished. The first group comprises the basic amino-acids arginine, histidine and lysine, the second leucine and isoleucine and the third glycine, alanine, glutamic acid and creatine. The sugars glucose and xylose form one group, possibly loosely related to another group, containing fructose and galactose.

Such a loose connexion between groups has been interpreted on the assumption that certain features of the transport mechanisms are shared by two groups, others not. Gammeltoft & Kjerulf-Jensen visualized the common feature of fructose and galactose on the one hand and of glucose and xylose on the other as either a common phosphate donor or a common source of energy.

In the case of tubular secretion likewise competition phenomena are common. T_m of phenol red is lowered by diodrast as well as by hippuran, whereas phenol red is much less effective in inhibiting the secretion of diodrast and hippuran, indicating transport by a common mechanism, but with differences in affinity.

The secretion of a number of organic bases, including methyl-nicotinamide, is not inhibited by diodrast or *p*-amino-hippuric acid. Thus at least two different transport mechanisms appear to exist with respect to tubular secretion.

(4) *Enzyme inhibitors*

The absorption of glucose from the intestine as well as from the kidney tubules is depressed by phlorizine (Nakazawa, 1922; Lundsgaard, 1933*a*; Wertheimer, 1933; Walker & Hudson, 1937), in the case of the kidney completely. Phlorizine, however, was also found to inhibit the tubular excretion of phenol red in man (Chasis, Ranges, Goldring & Smith, 1938) and in the chicken (Pitts, 1938), as well as that of diodrast in man (Chasis *et al.* 1938). Cyanide inhibits the absorption of glucose (Kjerulf-Jensen & Lundsgaard 1940). The reabsorption of sugars in the kidney was found by Höber (1933) to be depressed by phenyl urethane. Iodoacetic acid inhibits glucose absorption both from the intestine (Wilbrandt & Laszt, 1933) and from the tubule (Walker & Hudson, 1937), as well as secretion of phenol red in the chicken mesonephros (Beck & Chambers, 1935).

The assumption that enzymatic reactions are somehow involved in active transport and that depression of a transport by enzyme inhibitors may be taken as a criterion for the active nature of the transport has rather generally been made, and, in fact, can hardly be doubted.

The question, however, arises in each case and has much been discussed as to whether an action of an inhibitor has to be interpreted, roughly speaking, as blocking the machine itself or the burning of the fuel, i.e. whether it is specifically related to a reaction involved in the transport mechanism of the substance in question or whether it inhibits energy-supplying reactions that could be used for other transports likewise or even for any kind of work done by the cell. Inhibitors affecting the transport directly may be termed *primary inhibitors*, those acting on the energy metabolism *secondary inhibitors*.

Enzyme inhibitors of high specificity are not among those listed above. The majority would clearly be classed as secondary inhibitors. For a time there seemed to be evidence for a primary action of phlorizine on phosphorylation processes involved in glucose transport. Lundsgaard (1933*b*) showed that the uptake of inorganic phosphate by intestinal mucosa was inhibited by phlorizine. This, however, may have been due to the inhibition of phosphorylase. If there is a primary inhibition by phlorizine it would appear to be more likely an inhibition of phosphatase. But the inhibition of various secretion processes in the kidney tubule mentioned above, as well as the inhibition of dehydrogenases by phlorizine shown by Shapiro (1940, 1947), would be equally reconcilable with a secondary action, as this author pointed out. One point in favour of a primary action appears to be the effect on the glucose transport through the red cell membrane which will be referred to later, because in this case an 'uphill' transport has not been demonstrated and a requirement of energy-supplying reactions seems doubtful.

Thus the particular role of enzymes in the transports considered here has not been elucidated to a considerable extent by the study of enzyme inhibitors so far. This situation may change, however, if effective inhibitors of higher specificity should be found.

III. THE TRANSPORT MECHANISM

In the mechanism of transport enzymatic reactions involving the transport substrate may be assumed which somehow affect the overall rate of diffusion. This linking to diffusion rate has been visualized repeatedly in the form of a combination with a carrier to form a substrate carrier complex, by which either diffusion in the direction of the transport is enhanced or back-diffusion is inhibited. Mainly two such mechanisms have been suggested which may be called the cytoplasm-carrier mechanism and the membrane-carrier mechanism.

(1) *The cytoplasm-carrier mechanism*

If the substrate S after passage of the membrane by diffusion combines with a carrier C to form a complex CS, under certain conditions the rate of diffusion may be increased. Such a reaction was first suggested by Höber in 1899 to account for the relatively rapid sugar absorption from the intestine. Verzàr (1931) proposed the same principle. The acceleration was ascribed to the maintenance of a steep concentration gradient across the membrane due to elimination of the substrate beyond the membrane by the reaction assumed. As Danielli (1943) rightly pointed out, this acceleration is only possible if CS does not (or only much slower than S) penetrate the membrane.

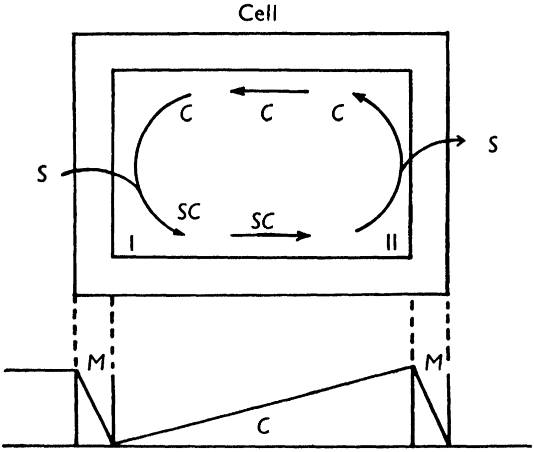

Fig. 4. Schematic picture of the cytoplasm carrier mechanism. C = carrier; S = substrate; M = membrane; Cy = cytoplasm. I and II: sites of the chemical reactions assumed. Below: gradients for the substrate.

Later a second reaction releasing the substrate again at the other end of the cell was introduced in the mechanism suggested by Shannon & Fisher (1938) and by Kalckar (1937) and others. A schematic picture of the mechanism is given in Fig. 4. According to this view the substrate S would pass the first cell membrane in free form, the cytoplasm in the form of CS and the second membrane again in the form of S. Drabkin (1948) assumed hexokinase to be enzyme I and phosphatase to be enzyme II.

In principle this mechanism has been widely accepted and used to explain a series of observations. The limited amount of C (whose concentration would be the extensive factor of the scheme) gives a basis for the interpretation of maximum rates as well as of competition. The assumption of different dissociation constants of the complex CS was successfully used to explain differences of T_m values for various compounds and special features of competition, particularly the fact that competition may be asymmetric in the sense that A competes more powerfully with B than vice versa (e.g. phenol red and diodrast, as mentioned above). The complex constant would be the intensive factor of the scheme.

There are, however, a number of difficulties for the acceptance of the particular assumption that the carrier substrate complex is formed inside the cell.

For the discussion of the potentialities in this scheme it seems useful to introduce a value for the permeability of the membrane. Since, irrespective of factors like lipoid solubility and porous structure of the membrane, the rate of diffusion will always be proportional to the concentration difference across the membrane, this may be done in the form of a 'diffusion-equivalent thickness' of the membrane, d_e—the thickness of a water layer through which transport by free diffusion would occur with the same rate as it actually does through the membrane (assuming equal concentration difference). If the permeability constant P is expressed in cm./sec. and the diffusion constant D in cm.^{2}/sec., d_e in cm. will be given by D/P.

If the dimensions of membrane and cytoplasm are chosen according to their values of d_e, the concentration gradients in the steady state will be parallel as shown in Fig. 5.

Three cases have been represented in this figure:

A, 'Downhill transport' with small (A_1) and with large (A_2) values of d_e,

B, transport between equal concentrations, and

C, 'uphill transport'.

The reactions assumed are termed I (formation of CS) and II (releasing of S from CS).

Under the conditions of A the reactions merely serve to accelerate diffusion as pictured by Höber and by Verzàr. The acceleration ratio has a maximum value of 2 if the concentration of S by the reaction I is kept near zero (as assumed in Fig. 5).

Under the conditions of B and C the transport would not occur spontaneously without reactions I and II (acceleration ratio $= \infty$ or negative).

The presupposition for both B and C is a sufficiently high free energy of reactions I and II. The gradients in Fig. 5 have been drawn for high values of ΔF, such that the concentrations of S and CS are kept near zero by I and II respectively. This results in maximum steepness of the gradients. If the free energies are lower, these concentrations will rise, the gradients become less steep and the rate of transport will decrease.

The maximum rate will be given by the maximum slope of the gradients (as in Fig. 4), i.e. it will be determined by the substrate concentration S_{I}

before passage of the first membrane, divided by d_e of the membrane. This means proportionality between S_{I} and rate of transport for low concentrations. At high concentrations the rate will be limited by the concentration and turn-over numbers of the enzymes for I and II. It will, then, attain a constant level in the cases B and C, but not in the case A because of continuing diffusion of free S.

This difference between conditions A and B or C might be used to explain the observation that a constant maximal rate of transport was experimentally reached in general in the kidney tubules (where due to the filtration process the conditions of B hold), but not in the intestine (where the experimentally used concentrations in the lumen usually were considerably higher than the plasma concentrations according to condition A). It is, however, at variance with observations on the aglomerular kidney, where the condition A prevails (for high concentrations) and nevertheless a definite maximal rate of transport for phenol red was observed.

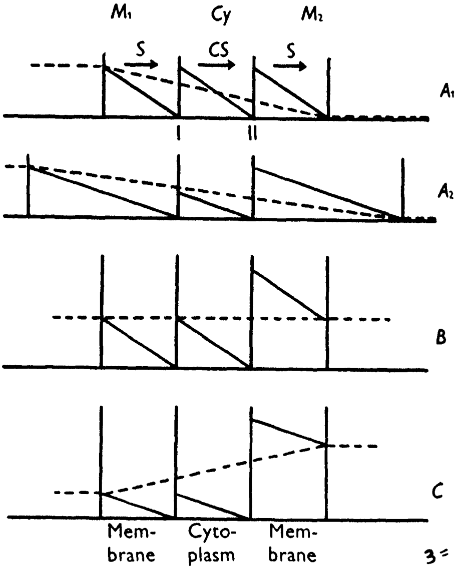

Fig. 5. Gradients for the substrate in the cytoplasm carrier mechanism, taking the thickness of the membrane as its diffusion equivalent value. (Dashed lines: gradients when reactions I and II are blocked).

Being given that the rate of transport at low concentrations is limited by the permeability of the membrane ($1/d_e$), a maximum value for d_e may be derived from the amount of glucose reabsorbed in man per unit time, T. It was shown above that the amount transported through unit area (cm.^{2}) in the tubule may be approximately estimated to be $0{\cdot}21 \times 10^{-8}$ mol./sec.

This rate, according to Fig. 2, is still held at a glomerular concentration of 200 mg.%, i.e. an average tubular concentration of 100 mg.% or 0·006 molar $= 6 \times 10^{-6}$ mol./ml. Thus assuming penetration through the membrane by diffusion of free glucose, a minimum permeability constant $P = 3{\cdot}5 \times 10^{-4}$ cm./sec. would result. (This is a minimal value, since it only holds for zero concentration within the cell.) The diffusion constant D being about 10^{-10} cm.^{2}/sec., for d_e a maximum value of 27 Å. would be arrived at. Since the thickness of the cell membrane is estimated to be about 100–200 Å., this result would mean that glucose penetrates the membrane 4–8 times faster than in free diffusion which, of course, is absurd. If, furthermore, the thickness of the brush border (about 20,000 Å.) is assumed to add to the value of d_e, which seems a reasonable conclusion, the discrepancy becomes even 100–200 times greater.

In addition, it should be recalled here that as discussed above in general the substances transported actively are not substances which penetrate easily.

Inhibition of the reactions I and II would have different effects according to the direction and value of the concentration difference. In the case A the transport would not be blocked but slowed down, in the case B it would be blocked, in the case C reversed. Again this would appear in harmony with some observations, but not with others. In the intestine (condition A), phlorizine in general only diminished the rate of absorption of sugars, in the kidney (condition B or C) it seems to block it completely. In the double perfusion experiments of Höber (1933), however, on the frog kidney application of phlorizine and glucose to the tubules but not to the glomeruli never caused glucose to appear in the urine, as it should be expected, if the tubule cell membranes were permeable to glucose.

Thus serious difficulties arise when the implication of the cytoplasm-carrier mechanism are considered in detail and quantitatively. This was one of the reasons for the suggestion of the membrane-carrier mechanism by Rosenberg & Wilbrandt (1952). In this mechanism, which shares the useful possibilities of interpretation mentioned above with the cytoplasm-carrier mechanism, the features just discussed offer no difficulties.

(2) *The membrane-carrier mechanism*

In this mechanism, which is represented in Fig. 6, the carrier substrate complex is assumed to be formed before the passage of the first membrane, and, in fact, to be the pre-condition of this passage, the membrane being assumed to be practically speaking impermeable to the substrate. The complex thus would differ from the substrate in the ability to penetrate the membrane, possibly due to factors like lipoid solubility.

The difficulties just discussed are avoided by this interpretation.

If the membrane is impermeable to the substrate itself, blocking of the mechanism will stop the transport completely, independent of the concentration conditions. This is in accordance not only with the observations of Höber mentioned above, but also with data on red cells to be discussed later.

The rate of transport will be determined by the diffusion rate of the complex rather than of the substrate. Thus the assumption of impossibly high permeability constants will not be necessary, since the concentration of the complex may be raised by reaction I (now at the outer surface of the membrane) to high levels, if its free energy is sufficient.

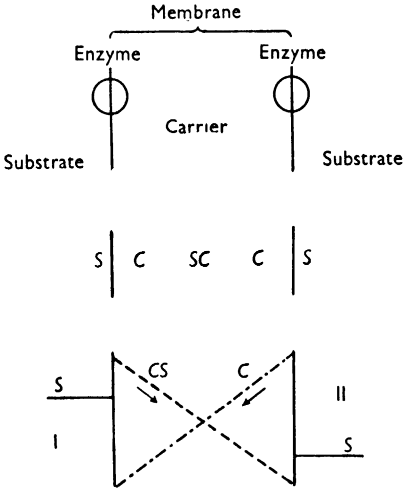

Fig. 6. Scheme of the membrane carrier mechanism. The enzymes sited on the two surfaces of the membrane catalyse the substrate carrier reactions shown in the middle and thus build up the gradients shown below.

Finally, the maximum rate, limited by saturation of the carrier (or of the enzyme catalysing the substrate-carrier reaction), will under all conditions be independent of the substrate concentration, since no diffusion except that of the complex occurs in the membrane.

A further point in which the membrane-carrier mechanism appears superior to the cytoplasm mechanism is the efficiency of the system with respect to back-diffusion. In the cytoplasm-carrier scheme back-diffusion through the first membrane can be avoided, if reaction I is sufficiently rapid and if *CS*, as mentioned above, does not penetrate the membrane. Back-diffusion of *S* through the cytoplasm from the site of reaction II to that of reaction I, however, appears to be inevitable. Reaction I, thus, not only will have the task of removing *S* coming through the membrane in the direction of the transport, but also *S* coming back from the site of reaction II. The ratio of these fractions which may be called 'back-diffusion efficiency' depends on both d_e and accumulation ratio. If the accumulation ratio is a, and the ratio of the length l of the cell to the equivalent membrane thickness l/d_e is b, the back-diffusion efficiency, e, can be shown to be

$$e = \frac{b}{b + l + a}. \tag{5}$$

Remembering that actively transported substances in general penetrate cell membranes slowly, we may take d_e of a poorly penetrating substance, for instance, glycerol in the ox erythrocyte, for which P, according to Jacobs (1934), is $0{\cdot}002 \times 10^{-5}$ cm./sec. Taking for D $0{\cdot}812 \times 10^{-10}$ cm.^{2}/sec. we obtain a value for d_e of $40{\cdot}6\,\mu$. If the length of the cell is taken as $20\,\mu$, b will be 0·5. For an accumulation ratio of 100, which is not infrequent, the efficiency then becomes 0·5/101·5, i.e. less than ½%.

Another reason for preferring the interpretation of the membrane-carrier mechanism, however, was the fact that with the exception of uphill transport, all the features of active transport discussed above could be demonstrated in the case of glucose transport through the red cell membrane. These results, which were obtained in collaboration with Rosenberg (many of them also independently by LeFevre (1947, 1948) and LeFevre & Davies (1951)) will be discussed in the following section.

IV. THE TRANSPORT OF SUGARS ACROSS THE RED CELL MEMBRANE

The membrane of red cells in most species has a very low permeability for monosaccharides; in various species it is practically speaking impermeable. The erythrocyte of man and apes, however, as was early shown by Kozawa (1914), are highly permeable.

The transport leads to equalization of the concentration. Up to now accumulation has not been demonstrated in a conclusive way.

In other respects, however, striking similarities were found to the transports in kidney and intestine.

Phlorizine was found to inhibit the penetration of glucose, fructose, D-xylose and L-arabinose (LeFevre, 1947; Wilbrandt, 1947, 1953). Iodoacetic acid, however, in contrast to its inhibition of the 'uphill' absorption in the intestine showed no effect (Wilbrandt, Guensberg & Lauener, 1947). This would seem to indicate that phlorizine actually is an inhibitor of the primary type, iodoacetate a secondary one.

The inhibition is also shown by phloretin, the aglucone of phlorizine, as well as by various phosphate esters of phloretin which will be referred to later (Wilbrandt & Rosenberg, 1950).

In view of the fact that hexokinase has been named as the first enzyme in the mechanism of intestinal and tubular absorption of glucose (Drabkin, 1948; Hele, 1950), lachrymators were tested whose hexokinase-inhibiting action has been shown by Dixon (1948). It was found that allylisothiocyanate, bromacetophenone and chloropicrine block glucose transport completely. ATP, however, and magnesium never accelerated but rather showed slight inhibition. The penetration of glycerol was not affected by lachrymators (Wilbrandt & Rosenberg, 1951; Wilbrandt, 1953).

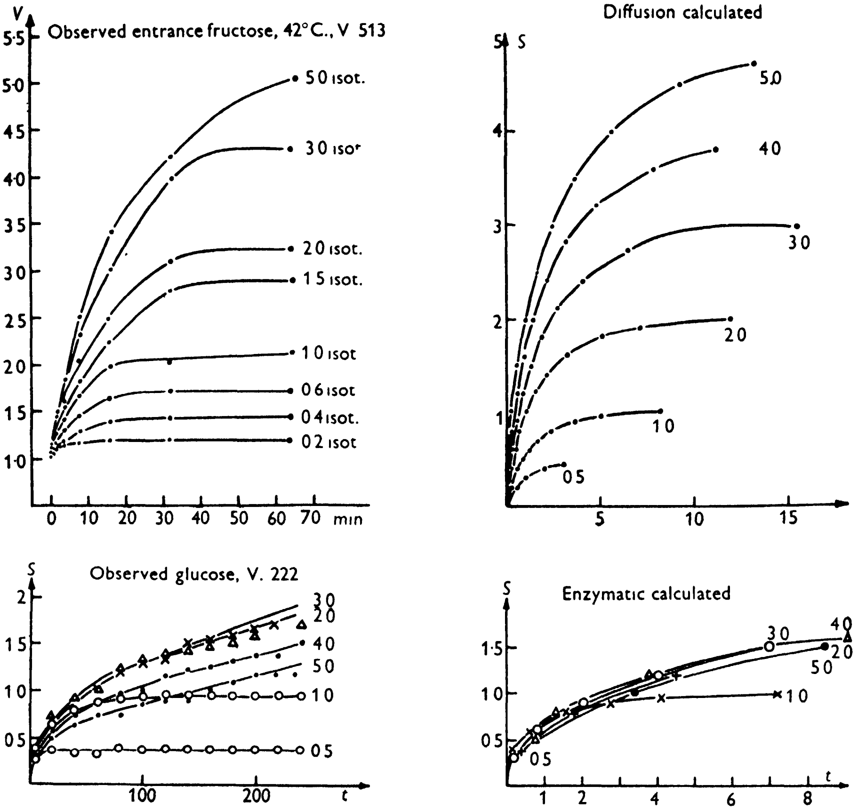

Fig. 7. Observed entrance of glucose and of fructose into human red cells from varied external concentrations as compared to the course of penetration calculated on the basis of the E. kinetics (see p. 157).

Corticosteroids which lower T_m for glucose in the human kidney were found to decrease the rate of transport (Wilbrandt, 1953).

Other inhibitors were heavy metals (LeFevre, 1948; Wilbrandt & Rosenberg, 1950), gold and mercury as well as chloromercuribenzoate (LeFevre, 1948), pointing to a probable role of SH groups.

No effect was observed with azide, dinitriphenol, atabrine, thyroxine, whose inhibiting or enhancing action on other transports most likely will be of the secondary type.

Insulin showed, if any, a slight inhibitory effect.

The simultaneous penetration of D-xylose, L-arabinose and glucose was much slower than the sum of the individual transport rates in the same concentrations, indicating competition for a common transport mechanism (Wilbrandt, 1950).

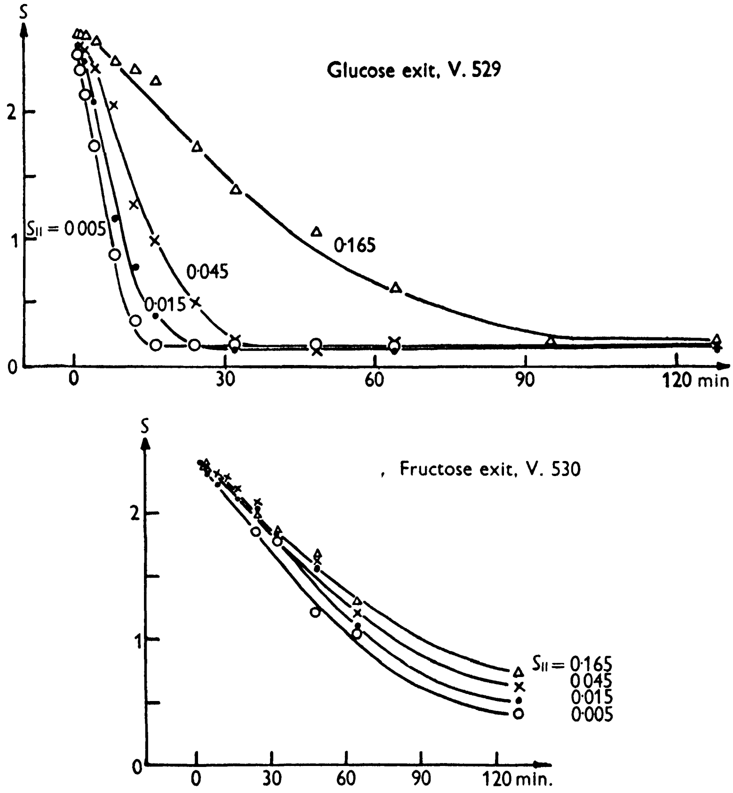

Fig. 8. Observed exit of gucose and of fructose from human red cells. S = amount of sugar in the cell in cell units (normal cell volume × isotonicity = 1).

Competition between various monosaccharides successively added has been investigated extensively by LeFevre & Davies (1951). They showed that the inhibition may be unilateral, e.g. the uptake of laevulose is strongly inhibited by glucose but not vice versa. Our own results agree closely with this observation. The authors also pointed out that certain differences in kinetics appear to parallel the behaviour with respect to mutual inhibition. The nature of these differences will be referred to later.

The dependence of the transport rate on the sugar concentration revealed unusual features, when studied in experiments involving both entrance and exit of glucose. For the sake of clarity the side where glucose enters the membrane was termed *cis*, that where glucose leaves the membrane *trans*.

With rising *cis* concentrations of glucose the rate increased initially and finally reached a constant level. This level, however, depended strongly on the *trans* concentration, even (or rather particular strongly so) in very low ranges of concentration (where the slope of a diffusion gradient would hardly be affected). Figs. 7 and 8 show such experiments.

This surprising feature led to an attempt to calculate the kinetics of an enzymatic membrane-carrier transport (Wilbrandt & Rosenberg, 1951). It was assumed that a first enzyme on the *cis* side catalyses the substrate-carrier reaction, that the complex diffuses across the membrane and that on the *trans* side it dissociates, catalysed by a second enzyme. Using the Michaelis-Menten equation for the rates of the enzymatic reactions and assuming a steady state, an equation for the rate of transport was arrived at. It can be resolved into separate terms, if instead of the rate of transport A its reciprocal $1/A$ is used (representing some sort of penetration resistance):

$$\frac{1}{A}=\frac{1}{E}\left(\underset{(1)}{\frac{K(S_{\mathrm{I}}+S_{\mathrm{II}})+2S_{\mathrm{I}}S_{\mathrm{II}}}{K(S_{\mathrm{I}}-S_{\mathrm{II}})}\frac{1}{K_2}}+\underset{(2)}{\frac{K(S_{\mathrm{I}}+S_{\mathrm{II}})+2K^2}{K(S_{\mathrm{I}}-S_{\mathrm{II}})}\frac{1}{K_1}}\right)$$

$$+\frac{1}{M}\left(\underset{(3)}{\frac{S_{\mathrm{I}}S_{\mathrm{II}}}{S_{\mathrm{I}}-S_{\mathrm{II}}}\frac{1}{K}\frac{K_1}{K_2}}+\underset{(4)}{\frac{S_{\mathrm{I}}+S_{\mathrm{II}}}{S_{\mathrm{I}}-S_{\mathrm{II}}}}+\underset{(5)}{\frac{1}{S_{\mathrm{I}}-S_{\mathrm{II}}}K\frac{K_2}{K_1}}\right), \quad (5)$$

where A = rate of transport,

S_{I} = *cis* concentration of substrate,

S_{II} = *trans* concentration of substrate,

$C_{\mathrm{tot.}}$ = total concentration of carrier (free and bound),

D = diffusion constant of the carrier (assumed to be equal to that of the carrier-substrate complex),

M = $C_{\mathrm{tot.}}\, D$,

E = concentration of enzyme (assumed to be equal *cis* and *trans*),

K = Michaelis-Menten constant (likewise assumed equal *cis* and *trans*),

K_1 and K_2 = velocity constants of the enzymatic reaction and back-reaction between substrate and carrier.

Of the terms (1), (2), (3) and (5) any one may be limiting, according to the ratios of the various constants and concentrations, as shown in the following table:

Limiting terms and forms of penetration kinetics

				$K > S_{\rm I}, S_{\rm II}$	$S_{\rm I} > K > S_{\rm II}$	$S_{\rm I}, S_{\rm II} >$
			Substrate... concentration			
			Enzymes...	Not saturated	$E_{\rm I}$ saturated $E_{\rm II}$ not saturated	Saturated
	Limiting term	K_2/K_1	Carrier			
E limiting	1	$\ll 1$	Saturated	Z	Z_1	E
	2	$\gg 1$	Not saturated	D	Z_1	Z
M limiting	3	$\ll 1$	Saturated	E	E	E
	5	$\gg 1$	Not saturated	D	D	D

Thus five types of kinetics result which have been termed D, Z_1, Z_2, Z and E and whose characteristics are shown in Fig. 9. D is the kinetics type of free diffusion.*

It appears that under conditions under which either the enzymes or the carrier or both are saturated the type E will frequently be found. This, however, is the only type harmonizing with the observations reported above for glucose.

On the other hand, the type D, i.e. a behaviour like that of free diffusion, may also be met, particularly if the carrier is not saturated.

This agrees well with the observation that for fructose, although its transport is also inhibited by phlorizine and thus may be assumed to be based on the same essential mechanism as that of glucose, the striking dependence of the rate of transport on both *cis* and *trans* concentrations is not observed. It seems rather to follow the D type (see Figs. 7 and 8). Accordingly the affinity of fructose to enzyme or carrier may be assumed to be lower than that of glucose.

This difference in affinity as concluded from the kinetics, on the other hand, is in good harmony with the observation reported above that glucose saturation depressed fructose transport much more than vice versa.

The course of penetration for the E kinetics has been calculated both for entrance and exit and showed close resemblance to the observed course for glucose. In Fig. 7 calculated and observed curves for entrance are represented.

In the calculation of equation (5) for the sake of simplicity the assumption was made that enzymes I or II have the same Michaelis constant, or that they are identical. This, however, is not a necessary requirement. In fact, experiments with phloretin phosphate strongly indicate that in the red cell the enzymes are different.

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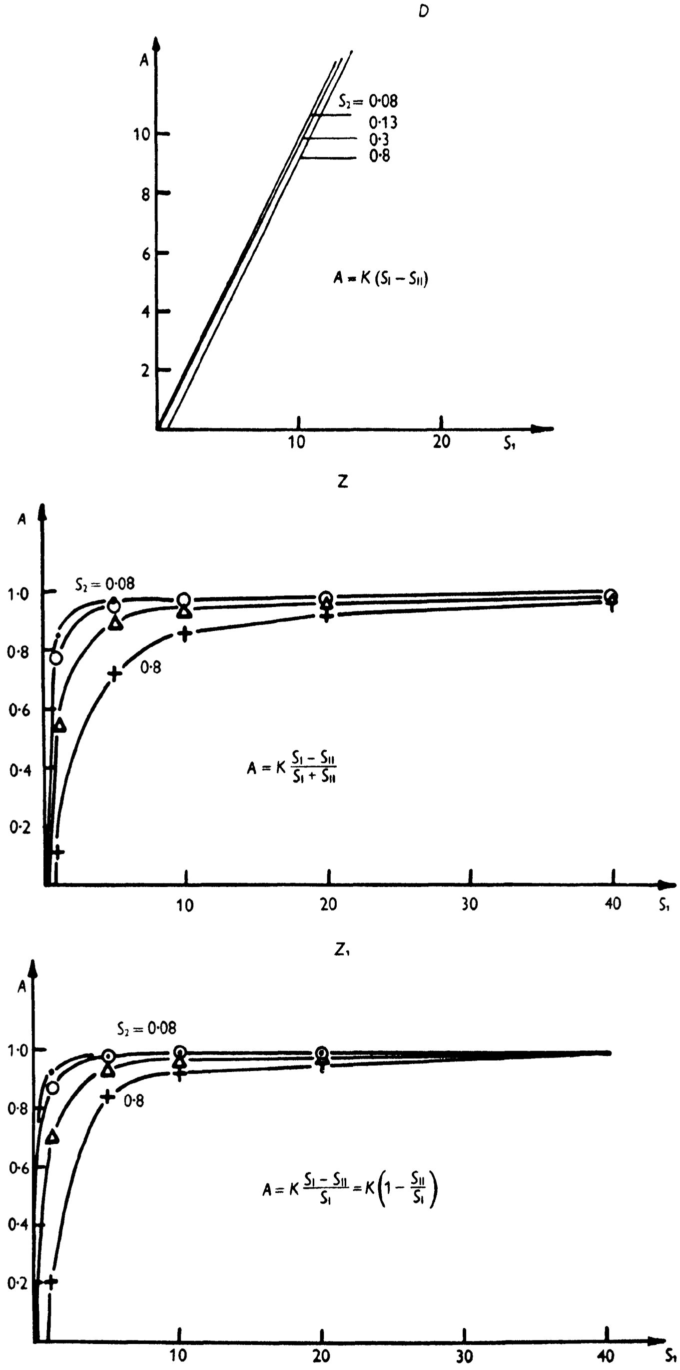
* Z_2 only results if either the enzyme concentrations or the velocity constants on the *cis* and the *trans* side are different (which was not assumed here). The general equation for this case was given previously (Wilbrandt & Rosenberg, 1951).



For legend see p. 159.

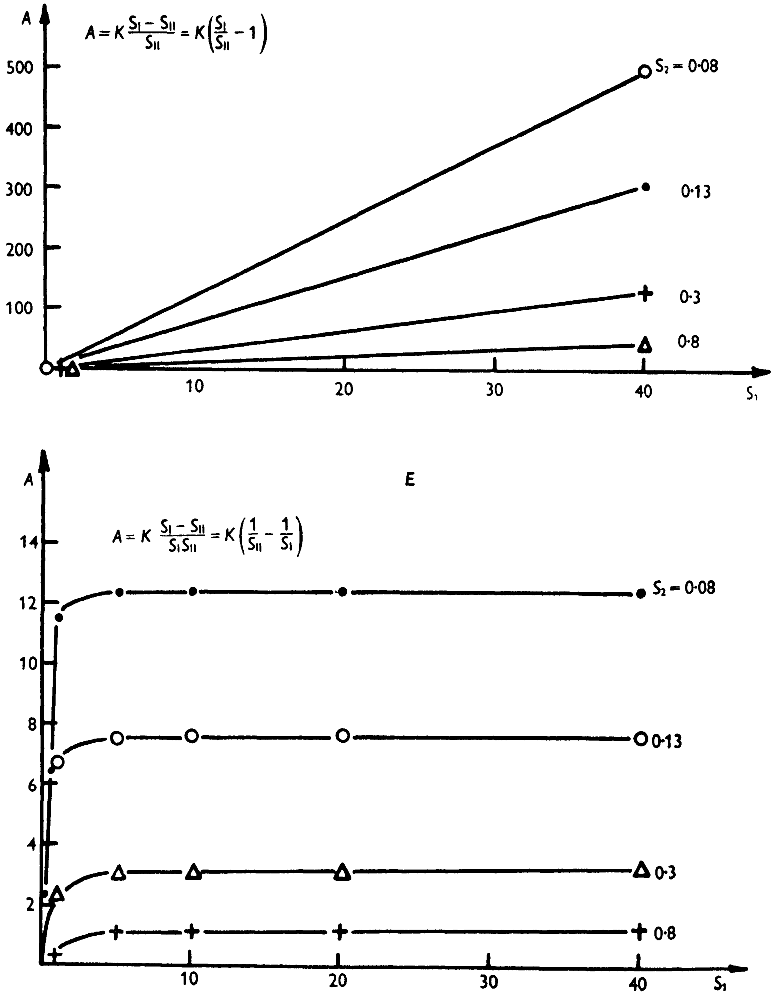


Fig. 9. Types of penetration kinetics resulting from equation 5, when certain terms become limiting (see text).

The phloretin phosphate preparations used were highly polymerized, and a ^{32}P labelled specimen could be shown not to penetrate the red cells to any appreciable extent. Thus the action of this inhibitor will be restricted to the outer surface of the membrane.

The inhibition by these phloretin esters, in striking contrast to most of the other inhibitors tested, showed a most definite preference for the glucose exit, as compared to the entrance. In some experiments, under strictly parallel conditions, the entrance seems not to be inhibited at all, whereas the exit was blocked for a long time.

Phlorizine showed qualitatively similar behaviour; inhibition of glucose entrance, however, increased with time, most likely indicating slow penetration of phlorizine.

Several conclusions may be drawn from these results: there must be at least two enzymes on the outer surface of the membrane, only one of which is inhibited by phloretin phosphate, and the inhibited enzyme must be used preferentially for outward transport, the other one for inward transport. The kinetics of inhibition of the transport by various inhibitor types (both competitive and non-competitive) has now been worked out. Marked kinetical asymmetries may occur, even with identical enzymes *cis* and *trans* and varying with the type of inhibitor. Thus the question whether different enzymes *cis* and *trans* have to be assumed must be left open at present. If some sort of a phosphorylation-dephosphorylation mechanism is involved, as has been assumed for the epithelial transports, it must be the dephosphorylation stage that is inhibited by the phlorizine group, as suggested above.

This would appear in harmony with the results of Ellinger & Lambrechts (1937) on various azo derivatives of phlorizine. Only those compounds inhibited glucose reabsorption that could be shown to penetrate the tubule cells.

Such a phosphorylation mechanism, however, cannot be as simple as has been assumed. Glucose-1-phosphate, as well as glucose-6-phosphate and fructose diphosphate, have been tested and found not to penetrate the red cell membrane appreciably. Formation of a metaphosphate ester has been suggested based on several theoretical considerations (Rosenberg, 1948, 1950), but no way of experimental test has yet been found.

SUMMARY

A survey is given of active transports of organic molecules through the epithelia of intestine and kidney.

It is shown that most of the substances actively transported either are known to or may be assumed to penetrate cell membranes only very slowly, and that a polar-non-polar structure of the molecules appears to favour active transport in the case of secretion.

Examples are given for the following characteristic features of these transports:

(1) The majority of them occur or may occur against a gradient of electrochemical potential, requiring coupling with energy supplying reactions.

(2) Enzyme inhibitors affecting the transports may be classed as primary inhibitors acting on reactions involved in the transport mechanism itself

and secondary inhibitors, affecting energy-supplying reactions. Few if any primary inhibitors have become known.

(3) Competition for the transport mechanism among molecules of like structure is frequent. In the kidney several groups of such potentially competing molecules have become known.

(4) The rate of transport shows in general a non-linear relationship to the concentration, in contrast to diffusion. In many cases with high concentrations a maximum constant level of the rate is attained.

Two carrier mechanisms that have been suggested are compared, one of them assuming the formation of a substrate carrier complex after, the other one before passing of the first cell membrane. The terms cytoplasm-carrier mechanism and membrane-carrier mechanism are suggested.

Arguments against the cytoplasm-carrier mechanism are considered which are based on consequences of the assumed free permeability of the cell membrane for the substrate: the general character of molecules actively transported, the facts that inhibition of the mechanism does not seem to lead to leakage, that to account for the maximal reabsorption of glucose in the human kidney, impossibly high permeability constants must be assumed and that due to back-diffusion of substrate in the cytoplasm the efficiency of the mechanism at high accumulation ratios would be very low.

Furthermore, the assumption of the membrane-carrier mechanism seems to be favoured by the fact that most of the above-mentioned features of active transport except the 'uphill' shift are found in the transport of sugars across the red cell membrane; depression by enzyme inhibitors like phlorizine and numerous others, competition among different sugars, non-linear rate-concentration dependency with a constant maximal rate level at high concentrations. The kinetics of a membrane-carrier mechanism have been calculated and found to agree satisfactorily with the observed data, particularly with respect to a striking dependence of the rate of transport on small concentrations of glucose on the *trans* side of the membrane.

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COMMENT ON PROFESSOR WILBRANDT'S AND DR LEFEVRE'S PAPERS

W. F. Widdas, London, said:

The general findings of Prof. Wilbrandt and of Dr LeFevre have been confirmed in experiments carried out on human erythrocytes and also on erythrocytes of a number of mammalian species (in which the cells from the blood of foetal and newborn animals have been shown to be permeable to glucose and other sugars).

In interpreting the results obtained, kinetics have been used which were based on a membrane-carrier mechanism such as Prof. Wilbrandt has described but in which it was postulated that the rate-determining step was the relatively slow movement of carriers and complexes between one interface and the other. It was assumed that the carriers remained in the interface sufficiently long to achieve, on average, adsorption equilibrium with glucose in the respective solutions.

It follows that the net transfer of such a system would be proportional to the difference in the fraction of carriers saturated with glucose at the two sides. These fractions can be represented by relationships of the Michaelis-Menten type. It can be shown that these kinetics reduce to a diffusion-type equation when the equilibrium constant is high, but when the equilibrium constant is low, relative to the concentrations used, the best approximation is to take the transfer rate as proportional to the difference in the reciprocals of the concentrations:

$$\underset{S \to S^1}{\text{Transfer rate}} \propto \left(\frac{1}{C^1} - \frac{1}{C}\right).$$

The diffusion equation applied to the red cell problem gives rise to an expression of the form

$$kt = [F(C, V)], \tag{1}$$

whereas the second approximation gives an expression of the form

$$k't = C^2\,[F'(C, V)]. \tag{2}$$

The terms in square brackets are not identical but are of the same order, and if one uses the diffusion equation to analyse data of swelling in glucose solutions the theory predicts that

$$k_{\text{diff.}} \propto \frac{1}{C_2},$$

or

$$\log k = \text{constant} - 2 \log C,$$

that is, a plot of log k against log C should have a slope of -2.

My results and those of Wilbrandt, Guensberg & Lauener (1947) agree well with this prediction (see Fig. 1).

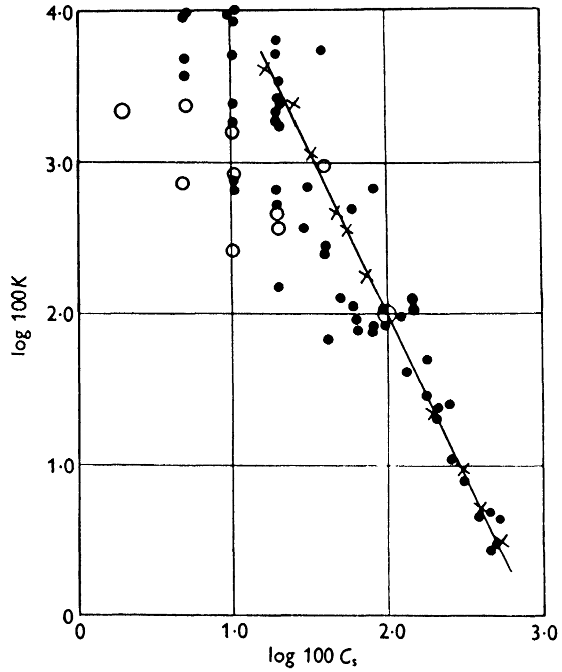

Fig. 1. Variation of apparent penetration constant (based on diffusion) with glucose concentration. Experimental results of Wilbrandt *et al.* showing plot of log 100 *k* against log 100 *C*. Points ○ and ● refer to results obtained by a direct and indirect photoelectric method respectively. Taking ⊕ as reference, points × represent calculated values; the slope of the line is approximately −2.

The kinetics have also been extended to cover competition to yield a method of determining the equilibrium constant of the carriers reacting with glucose (Widdas, 1953). The value reported agrees well with that obtained by Dr LeFevre by the phloretin method.

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ENZYME SYSTEMS OF THE CELL SURFACE INVOLVED IN THE UPTAKE OF SUGARS BY YEAST*

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I. INTRODUCTION

The hexoses constitute one of the most important sources of carbohydrate for heterotrophic organisms such as Protozoa, bacteria and fungi, as well as for most cells of multicellular animals. The enzymic systems by which sugars are assimilated and degraded have been worked out in considerable detail in cell-free systems, but a number of important questions remain unanswered when the uptake and metabolism of sugars by intact cells is considered—questions related to the architecture and organization of the cell. In recent years, with the development of techniques for isolating certain of the cellular structures such as mitochondria, chloroplasts, the nucleus and various granules of the cytoplasm, it has been clear that certain metabolic functions are localized in specific centres in the cell. In regard to sugar uptake, certain questions are pertinent. Where, in the cell, are the enzymes located which metabolize sugars? How does the sugar pass from the environment to the enzyme centre? All substances passing into the cell must pass through the cell membrane. What is the role of this structure in sugar uptake? In the past, it had generally been supposed that the cell membrane could regulate the rate of movement of glucose into the interior of the cell, the regulatory mechanism consisting of a resistance to the diffusion of glucose, which was defined in terms of a permeability constant. More recently there has been increasing evidence of participation of the membrane in an active transport of glucose into the cell, independent of the concentration gradient, and with the necessary energy supplied by metabolic reactions.

The permeability of cellular membranes to glucose has been the subject of considerable study since the classical work of Overton, Hedin and others in the late nineteenth century. There are a number of excellent reviews of this material (Brooks & Brooks, 1941; Davson & Danielli, 1943; Höber,

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* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N.Y.

1945; Heilbrunn, 1952). With the exception of the red blood cell of primates, all have been characterized as having a relatively low permeability to glucose and other sugars. The red blood cell of primates is a special case involving a mechanism by which glucose is actively transported across the membrane (LeFevre, this volume, and Wilbrandt, this volume). Permeability studies have been largely restricted to cells which do not actively metabolize glucose so that the osmotic equilibria could be determined. Whether the data obtained can be applied as an estimate of the permeability of membranes of cells which actively metabolize glucose is of course open to question. Ørskov (1945) studied the permeability of yeast to a variety of substances by measuring volume changes in the cells by optical or haematocrit measurements. According to his calculations, the cells have a relatively low but definite permeability to glucose, galactose, xylose and arabinose. However, the technique does not exclude osmotic effects associated with metabolism of these substances, nor does it indicate whether or not the substances are entering the cells, unaltered, by a diffusion mechanism. Brooks (1947) has summarized attempts to estimate the permeability of yeast cells based on rates of metabolism of this substrate. However, such attempts are somewhat inconclusive because of the assumptions that must be made in the calculations. Furthermore, the results must be compared with data from other cells which do not metabolize glucose.

Conway (1950*a*), in an interesting paper, has described the phase distributions of various substances in yeast suspensions. He found that galactose and arabinose do not distribute in the cellular water to any appreciable extent after an hour, indicating an exceedingly low permeability of the cellular membrane to these sugars. Using Conway's technique, it was found that the yeast membrane is also impermeable to sorbose (Rothstein & Meier, 1953). Since the uptake of glucose, of mannose, and of fructose is considerable under the same experimental conditions, it must be concluded that the yeast-cell membrane can discriminate between glucose, fructose and mannose as compared with galactose, sorbose and arabinose. Although the existing data do not unequivocally establish that the permeability of cell membranes is sufficiently high to account for the rate of uptake of glucose in yeast, the specificity of the yeast membrane in terms of its ability to pass glucose, fructose and mannose, but to reject galactose, sorbose and arabinose, argues against a simple permeability mechanism for the uptake of sugars.

On the other hand, evidence does suggest the existence of an active transport system for glucose. It has already been noted that the apparently anomalous permeability of the red blood cells of primates involves such a mechanism (LeFevre, this volume, and Wilbrandt, this volume). The movement of glucose from the lumen of the intestine across the epithelium into the blood also involves an active transport mechanism (Höber, 1945). In the renal tubule the glucose actually moves in the direction opposite to that which would be dictated by the concentration gradient (Smith, 1951). Although the energy for these transport mechanisms is undoubtedly derived from metabolic reactions, the exact mechanisms by which it is accomplished are not known. The demonstration of active transport mechanisms in the above-mentioned tissues does not necessarily permit the generalization that other actively metabolizing tissues and cells also depend on such mechanisms for supplies of sugar substrates.

II. AN HYPOTHESIS CONCERNING THE MECHANISM OF UPTAKE OF GLUCOSE BY YEAST

In this section studies are presented which are concerned with sugar uptake by yeast cells. These are not studies of permeability in the classical sense. Glucose as it enters the cell is altered by the metabolic cycles so that osmotic equilibrium is never attained. Therefore it is not feasible to determine concentration gradients, the rate of attainment of equilibrium, or the rates of inward and outward movements of glucose. Fortunately, it has been found that certain cations markedly influence the rate of uptake of glucose. Furthermore, the action of these cations can be localized at the periphery of the cell. The mechanism by which glucose proceeds through the membrane can therefore be characterized in terms of the effects of these substances on rates of glucose uptake. On the basis of these studies and of other evidence, an hypothesis concerning the mechanism of uptake of sugars can be proposed in general terms. It will be discussed in greater detail in the last part of this section. The hypothesis is based on the concept that glucose is actively transported into the yeast cell. It is furthermore suggested that this is accomplished by the presence of enzymic activity in the peripheral layers of the cell. The enzymic activity need not be a special mechanism for moving glucose into the cell, but may constitute the initial phosphorylation reactions of the fermentative and respiratory pathways of metabolism. Because of the peripheral location of the fermentative enzymes, glucose does not have to pass into the interior of the cell, though its metabolic products undoubtedly do so. The energy resulting from the peripheral degradation of glucose not only accounts for the uptake of carbohydrate by the cell, but also provides energy for the active transport of ions such as K^+, H^+, Mg^{++}, Ca^{++} and $H_2PO_4^-$.

The hypothesis requires that enzymes be present on the surface of the cell. Although considerable evidence has accumulated that enzymic activity at the surface of the cell is responsible for transport of various materials into the cell (Rosenberg & Wilbrandt, 1952; Danielli, 1952), the evidence in most cases is indirect. However, a few specific enzymes have been definitely localized on the surface of the yeast cell including invertase (Wilkes & Palmer, 1933), maltase and lactase (Myrbäck & Vasseur, 1943), trehalase (Myrbäck & Oertenblad, 1937), and a number of acid phosphatases (Rothstein & Meier, 1948, 1949). In each of these cases, a single reaction is involved. A given substrate is hydrolysed into specific products which can often be quantitatively recovered in the medium. The specific enzymes involved can be characterized *in vivo* without separation from the living cell, in terms of the end-products of the reaction.

The surface reactions in glucose uptake cannot be so readily characterized. The products of the initial reactions at the surface of the cell are not recoverable in the medium. They serve as substrates for a series of reactions leading to end-products such as CO_2 and alcohol. Although the overall rate of the entire system of reactions can be readily stated in terms either of glucose consumption, or CO_2 production, or O_2 consumption, inability to characterize the initial reactions in terms of their specific products is a severe handicap. It has been possible, however, to characterize the surface reactions in sugar uptake in terms of the effects of certain inhibiting and stimulating agents, each of which can be shown to act on the cell surface. On the basis of the results obtained, it has been possible to make informed guesses as to the specific reactions involved in terms of the known cycles of carbohydrate metabolism. The agents used include the following: UO_2^{++}, Mn^{++}, Ca^{++}, Mg^{++}, K^+, H^+, NH_4^+, $H_2PO_4^-$.

III. URANIUM ACTS ON THE CELL SURFACE

Uranium in the form of uranyl ion has been a very useful tool with which to explore certain properties of the surface of the yeast cell. Its usefulness stems primarily from its ability to form complexes with a variety of biochemical substances, particularly those containing phosphate or carboxyl groups, resulting in inhibition of systems in which such groups are essential. The metabolism of sugar by yeast is particularly sensitive to uranium (Booy, 1940; Rothstein, Frenkel & Larrabee, 1948; Barron, Muntz & Gasvoda, 1948).

When uranium plus glucose are added to a yeast suspension, there is no measurable delay in the onset of the inhibiting effect, nor is there any progressive increase or decrease in the inhibiting action. Apparently equilibrium is rapidly achieved between uranium and the cells. This was confirmed by actual measurements of uranium binding by the cells (Rothstein & Larrabee, 1948). There was as much uranium binding after 1 min. as after 30 min., indicating a rapid equilibration. After several hours, however, an additional small increment of uranium uptake was observed which was not accompanied by an increment in inhibition. The following discussion is concerned with the rapid phase of uranium uptake.

The minimal rate of penetration of uranium into cells necessary to account for the rapid phase of uptake would have to be $8{\cdot}5 \times 10^{-11}$ mol./min./cm.^{2} of surface. This seems very rapid considering that the concentration gradient of uranium did not exceed 1×10^{-5} M. The permeability constant to account for this rate of uptake would have to be $1{\cdot}4 \times 10^{-7}$ mol./cm.^{2} of surface/sec. for a concentration gradient of 1 mol./l. This is about the same magnitude as permeability of various cell membranes to water, and considerably higher than similar constants for cations of the monovalent series such as potassium (Brooks, 1941). Although there is little comparative data on rates of uptake of bivalent cations on which to base unequivocal conclusions, the calculated minimal value for UO_2^{++} would seem to be inordinately high in view of its relatively high positive charge and low mobility.

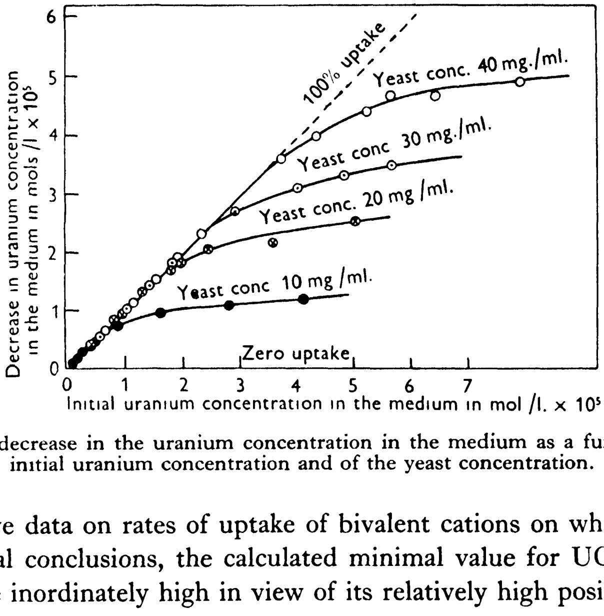

Fig. 1. The decrease in the uranium concentration in the medium as a function of the initial uranium concentration and of the yeast concentration.

The addition of increasing concentrations of uranium to a yeast suspension results in an increased uptake of uranium approaching a maximum value which is proportional in each case to the yeast concentration (Fig. 1), and amounts to 1×10^{-3} mol./l. of cells. In view of the fact that a maximum uranium uptake is observed, and in view of the fact that the distribution ratio between the cells and supernate is of the order of 100 to 1, it is evident that no simple distribution of uranyl ions between the medium and the cells takes place, but rather that certain constituents of the cell are binding the uranyl ion, and that the uranium-uptake curves represent saturation of these binding sites. Among those substances which are present in the cells which form relatively undissociated complexes with uranyl ion are the phosphates and the carboxyl-containing compounds, including bicarbonate, organic acids and proteins. The concentration of orthophosphates in the cell is $1\text{–}2 \times 10^{-2}$ M and of bicarbonate 1×10^{-1}. Thus the total uranium-binding capacity of the cytoplasm is at least 1×10^{-1} M/l. and considerably higher if proteins and organic phosphates are added, altogether well over 100 times the observed maximal uranium binding. It must be concluded that uranium equilibrates with constituents of the cell which are not in equilibrium with the cytoplasmic contents, but which are isolated by some barrier from the total uranium-binding substances of the cytoplasm. The only part of the cell with which uranium could combine without equilibrating with the aqueous phase of the cytoplasm is the membrane of the cell, and perhaps its immediately underlying structures.

Further evidence for the peripheral action of uranium is given by the following experiment. Cells are exposed to 2×10^{-5} uranium, resulting in a 90% inhibition of glucose uptake. The addition of any uranium complexing agent to the medium results in competition with the cell for the available uranium. Consequently the binding of uranium by the cell is reduced and the inhibition is likewise reduced. For example, the addition of 2×10^{-4} M-orthophosphate to the uranium-poisoned suspension of cells will reduce the inhibition from 91 to 30%. In view of the fact that the cytoplasm already contains orthophosphate in a concentration of 1×10^{-2} M, 50 times as high as that added to the medium, the uranium must be bound in a location in the cell which is accessible to the phosphate of the medium rather than the phosphate of the cytoplasm. If the uranium were acting inside the cell, the addition of relatively low concentrations of phosphate to the medium should have little effect because much higher concentrations of this ion are already present there. Only if the uranium were complexed on the surface of the cell could low concentrations of phosphate achieve the reversal of inhibition.

IV. THE CHEMICAL NATURE OF URANIUM BINDING SITES OF THE CELL SURFACE

The binding of uranium by yeast can be characterized by a simple reversible reaction of the form

$$U + Y \rightleftharpoons UY. \tag{1}$$

The reversible nature of the binding has been established by experiments in which uranium-complexing agents, such as phosphate or organic acids, are added to uranium-poisoned yeast. There results a reduction in uranium binding and in the inhibitory effect. Washing with very large quantities of distilled water will also achieve the same end. Equation (1) can be tested by putting it in the form of the mass law,

$$K=\frac{(U)(Y)}{(UY)}, \qquad (2)$$

and determining the constancy of K. Of the terms in equation (2), (U) is the equilibrium concentration of free uranyl ion in the medium. Values for (U) in many experiments are exceedingly low, in the range of 1×10^{-6} to 1×10^{-8} M. These were measured by electroplating and α-counting techniques using natural uranium enriched with ^{233}U which has considerably higher α-activity (Rothstein, Frenkel & Larrabee, 1949). The value for (UY), the uranium bound to the yeast cell, was determined by subtracting (U) from the total concentration of uranium added initially. The concentration of uranium-binding sites of the cell was estimated from the maximal binding of uranium, assuming that each site can bind one uranyl ion. The value for (Y) can then be calculated by subtracting (UY) from the total number of yeast sites. Data from a typical experiment is presented in Table 1. Over a 20-fold range of total uranium concentrations, the calculated K from equation (2) remains relatively constant. Other mass-law formulations based on different ratios of reactants do not give constants. Thus equations (1) and (2) are adequate descriptions of the binding of uranium by cell-surface sites (Rothstein *et al.* 1948).

Table 1. *Mass law constants for binding of uranium by yeast*

The yeast concentration was 20 mg./ml. in each case, equivalent to a concentration of binding sites (Y_T) of 2×10^{-5} M.

$U_T\times 10^{-6}$ M	$(U)\times 10^{-7}$ M	$(UY)\times 10^{-6}$ M	$(Y)\times 10^{-5}$ M	$K\times 10^{-7}$ M
0·8	0·18	0·78	1·92	4·4
1·6	0·26	1·57	1·84	3·0
2·4	0·49	2·35	1·77	3·7
3·2	0·75	3·13	1·69	4·1
4·0	0·86	3·91	1·61	3·5
4·8	1·08	4·69	1·53	3·5
6·0	1·47	5·85	1·41	3·5
6·8	1·71	6·63	1·34	3·4
8·0	2·89	7·71	1·23	4·6
16·0	14·40	14·60	0·54	5·3

An attempt has been made to determine the chemical nature of the uranium-binding sites of the cell, by comparing the properties of the yeast-uranium complex with the properties of other uranium complexes (Rothstein & Meier, 1951). A large series of uranium-complexing substances were studied. The test system contained a fixed amount of yeast, a fixed concentration of uranium, and a variable concentration of each complexing agent. With no complexing agent added, 95% of the uranium was bound to the cells and 5% was free in the medium. On the addition of a soluble complexing agent, a competition was effected between the cells and the complexing agent for the available uranium. The uranium combined with the complexing agent remained in the supernate. Consequently, as the concentration of complexing agent was increased, a greater percentage of the uranium was found in the supernate. Fig. 3 shows the distribution of uranium between cells and supernate with varying concentrations of a number of complexing agents. In most cases a curve obtains, in accordance with the mass law, assuming a 1 to 1 ratio of reactants. Exceptions are orthophosphate and glycerophosphate, which form a complex with a 1 to 2 ratio of uranium to phosphate. The relative affinities of the various substances for uranium can be expressed in terms of the concentration of each substance which is associated with 50% of the uranium in the medium (C^{50}). In the case of macromolecules, such as proteins and phosphate polymers, it is necessary to determine the concentration of uranium-binding sites per unit weight of material. This was accomplished with the proteins, by an equilibrium dialysis technique, and with the phosphates, by a colorimetric technique. For example, serum albumen has 18 sites per molecule. Highly polymerized desoxyribonucleic acid has 1 site for each 4 atoms of phosphorus.

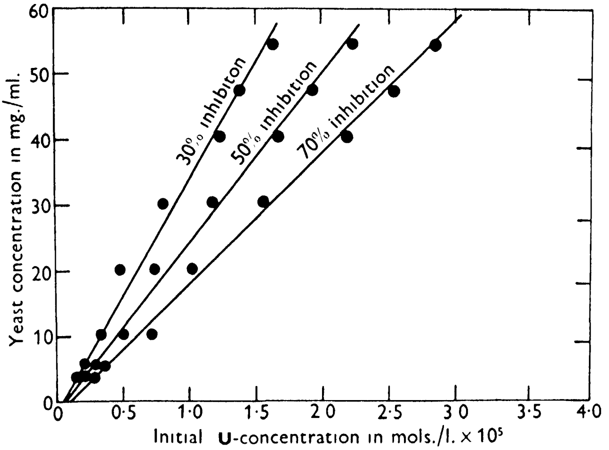

Fig. 2. Distribution of uranium between cells and supernate in the presence of various concentrations of complexing agents.

There was a wide spectrum of stabilities among the different complexing agents. C^{50}'s covered a 38,000-fold range. The weakest uranium complexors were the polyhydroxy compounds such as fructose. Among the organic acids, the monocarboxylic acids, such as acetate, formed relatively unstable complexes, whereas dicarboxylic acids, such as maleate, formed more stable complexes due to chelation. Citrate, a tricarboxylic acid, formed an even more stable complex due to multiple ring closure (Neuman, Havill & Feldman, 1951). The most stable carboxyl complexes were formed by the proteins. Here, accessory groups such as hydroxyl, perhaps imidazole and phosphate, may play an important role. Probably more than one carboxyl group is involved in binding each uranyl ion, for the number of uranyl ions firmly bound by each protein molecule represents only about one-fifth of the number of free carboxyl groups. Among the phosphates, compounds with a single phosphate formed relatively unstable complexes. As a class, compounds with multiphosphate structure, including polyphosphates and nucleic acids, formed the most stable complexes, with a stability increasing with the increasing molecular weight of the material.

Of all of the classes of agents tested, only the multiphosphate compounds gave complexes with a stability of the same order of magnitude as that of the yeast-uranium complex. The compounds which most resembled the cell in this respect were the metaphosphate polymers of high molecular weight. The polyphosphates of low molecular weight, such as ATP, showed a lower affinity for uranium by a factor of 20.

There has been a question raised concerning the validity of the mass-law equations in heterogeneous systems, that is, in systems containing both a solution and a solid phase (Rosenberg & Wilbrandt, 1952). Bonner, Argersinger & Davidson (1952) and Lowen, Stoenner, Argersinger, Jr., Davidson & Hume (1951) studied equilibria between cations and exchange resins. They found that the mass-law equations can be applied if the activity factors are taken into account, and that the true equilibrium constants can be obtained. Although the activity coefficients for the solid phases cannot be determined in an absolute sense, they are present in the mass-law equation in the form of a ratio which can be calculated from the data. For example, in equation (2) the two solid phases are present in the ratio $(Y)/(UY)$. If each term is multiplied by its activity coefficient, then the term becomes $\alpha_1(Y)/\alpha_2(UY)$. In the case of the cation-resin systems, the activity ratio, α_1/α_2, varies within an order of magnitude as the ratio $(Y)/(UY)$ is altered. However, when $(Y)/(UY)$ is 0·5, the ratio is approximately unity in the systems studied.

In comparative systems containing yeast, uranium, and a soluble complexing agent (C), the equilibria can be expressed as

$$\frac{K_y}{K_c} = \frac{\alpha_1(Y)}{\alpha_2(UY)} \frac{\alpha_3(UC)}{\alpha_4(C)}. \tag{3}$$

The comparison is made by determining the concentration of complexing agent which will result in a ratio of $(Y)/(UY)$ of 0·5. Although none of the activity coefficients is known for either the yeast groups or the complexors studied, the activity ratios α_1/α_2 and α_3/α_4 should cancel out, as an approximation. The fact that the highly polymerized compound (molecular weight about 2,000,000), in which the activity factors are comparable to those for yeast, possess the same affinity for uranium as does yeast, is strong evidence that the yeast surface sites are polyphosphates.

Can ATP be excluded as the binding agent on the surface of the cell? The stability of the ATP-uranium complex is one-twentieth that of the yeast-uranium complex. This difference would seem to be outside the error attributed to activity factors. However, another factor must be considered. Hurwitz (1953) has investigated the action of uranyl ions on the hexokinase-glucose-ATP-Mg^{++} system. He found that the hexokinase-ATP complex has a considerably greater affinity for uranyl ion than has free ATP, with consequent inhibition of the enzyme activity. The finding of Barron *et al.* (1948) that hexokinase was insensitive to uranyl ion was due to the presence of a large excess of ATP which forms a chelate with uranyl ion preventing it from acting on the enzyme. An ATP protein complex in the surface of the cell could therefore account for the affinity which the cell has for uranium.

Properties of the uranium complexes were also tested in regard to their stability as a function of hydrogen-ion concentration. The experimental technique was simple. A constant amount of yeast was mixed with a constant quantity of uranium. A series of such suspensions was adjusted to pH's in the range of 2·5–4·5. Higher pH's were avoided because above pH 4·5 uranyl ion forms a series of complexes with hydroxyl ion. With increasing pH there was an increased uptake of uranium complex. If the same experiment was repeated in the presence of a fixed amount of organic acid such as citrate, there was again with increasing pH an increased uptake of uranium by the cells. It is known that the organic acid complexes of uranium increase in stability as the pH is increased (Neuman *et al.* 1951), therefore it must be concluded that the yeast-uranium complex increases in stability to a greater extent than the carboxyl-uranium complex. If the experiment is repeated with a polyphosphate (hexametaphosphate) instead of citrate, then there is no redistribution of uranium as the pH is increased. In other words, the increased stability of the yeast-uranium complex is counteracted by an equal increase in the stability of the polyphosphate-uranium complex. Thus polyphosphates are chemically similar to the yeast surface sites not only because their uranium complex possess stabilities of the same order, but also because they are influenced by pH in the same manner. Carboxyl compounds do not resemble the cell surface on either score.

From the evidence cited above, it seems clear that the uranium binding of the yeast cell are multiphosphate in nature. However, on the basis of the existing techniques, it is impossible to determine the exact nature of the polyphosphate compound involved. It could be ATP, nucleic acid or metaphosphate polymer.

V. QUALITATIVE EFFECTS OF URANIUM ON METABOLISM

Low concentrations of uranium inhibit the fermentation of glucose, as measured by CO_2 production (Booy, 1940) as well as respiration of glucose, measured by oxygen consumption (Barron *et al.* 1948). Similar inhibitions are found if the disappearance of glucose is measured (Rothstein *et al.* 1948). Thus manometric measurements of oxygen consumption and CO_2 production can adequately characterize glucose uptake in the presence of uranium. In addition to its effect on glucose metabolism uranium also prevents the utilization of fructose. Yeast cells do not utilize galactose unless they have first been adapted to this substrate. Uranium blocks the utilization of galactose by adapted cells.

Uranium, even in relatively high concentrations, blocks only 90% of glucose uptake. Thus about 10% of glucose uptake proceeds through uranium-insensitive pathways. All of the studies reported here are concerned with the 90% of the sugar uptake which proceeds by uranium-sensitive pathways.

The action of uranium on metabolism is restricted to an inhibition of hexose metabolism (Rothstein *et al.* 1951). Thus uranium in sufficient concentrations to block sugar metabolism has no measurable effect on the respiration of alcohol and pyruvate, and only a small effect (less than 20% inhibition) on the respiration of acetate and lactate. Uranium in high concentrations has no effect on endogenous respiration as measured by oxygen consumption or glycogen depletion. Nor does uranium influence the synthesis of glycogen with alcohol as a substrate.

Normally yeast does not ferment its stores of glycogen, but in the presence of appropriate concentration of DNP, glycogen is rapidly degraded to alcohol and CO_2 (Rothstein & Berke, 1952). This DNP-induced endogenous fermentation is not inhibited by uranium.

In summary, then, uranium is an inhibitor of reactions specific to the uptake of the hexoses but is without effect on all of the metabolic pathways involved in the respiration and fermentation of other substrates, including stored glycogen. In terms of the generally accepted scheme of metabolism the action of uranium must be confined specifically to those reactions occurring at the cell surface which introduce sugars into the metabolic machine, without any effect on the integrity of the machine itself, which is presumably located inside the cell. The machine is therefore inaccessible to the action of uranium even though many of its component parts are inherently uranium-sensitive as shown by studies *in vitro* (Dounce & Lan, 1949).

The specific action of uranium on the initial reactions between glucose and the cell, and the insensitivity of the rest of the metabolic cycle, offers an explanation of the following phenomenon. If uranium is added before or at the same time as the glucose, the onset of inhibition is immediate. If, however, uranium is added 10 min. after the glucose, there is about a half-hour delay in the onset of inhibition. The delay is presumably associated with the utilization of intermediates of glucose which accumulated in the cell before the addition of uranium. Uranium prevents further glucose uptake, but does not interfere with the utilization of the intermediates formed from glucose.

VI. QUANTITATIVE ASPECTS OF THE ACTION OF URANIUM ON SUGAR METABOLISM

Two actions of uranium in yeast have been discussed: first, the formation of a stable complex with specific sites on the surface of the cell, and second, the inhibition of the uptake of hexoses by the cells. What is the relationship between the uranium binding and the inhibition of metabolism? The simplest relationship assumes that each surface site is directly associated with glucose uptake, and also assumes that the binding of a uranium molecule at that site prevents it from participating in glucose uptake. On the basis of this assumption there should be a direct proportionality between the number of sites which are combined with uranium, and the inhibition of metabolism. Thus if one-half of the available sites are combined with uranium, the inhibition should be 50% and with three-quarters of the sites bound the inhibition should be 75%, etc. Such a relationship can be expressed as

$$I=\frac{(UY)}{(Y_T)}, \tag{4}$$

or

$$1-I=\frac{(Y)}{(Y_T)}, \tag{5}$$

where I is the inhibition, (UY) is the concentration of uranium-bound sites, (Y) the concentration of unbound sites and (Y_T) the total concentration of yeast sites. Equations (4) and (5) can be tested experimentally by substitution in the mass-law formulation for the binding of uranium (equation (2)). Thus

$$K=(U)\frac{(1-I)}{I}. \tag{6}$$

Because the total uranium added (U_T), is equal to free uranium (U), plus bound uranium (UY),

$$K=[(U_T)-(UY)]\frac{1-I}{I}. \tag{7}$$

Again substituting equation (4),

$$(U_T)\frac{(1-I)}{I}=(Y_T)(1-I)+K. \tag{8}$$

But (Y_T) is proportional to the yeast concentration. Therefore

$$(U_T)\frac{(1-I)}{I}=kC(1-I)+K, \tag{9}$$

where C is the yeast concentration in mg./ml. and k is a conversion constant equating C and (Y_T). According to equation (9), at a fixed inhibition (U_T) should be proportional to C with an intercept equal to K. At 50% inhibition, the equation can be simplified to

$$(U_T)=0\cdot 5\ kC+K. \tag{10}$$

In Fig. 2 the (U_T) is plotted against C for 30, 50 and 70% inhibition. Data are taken from a series of curves at eight different yeast concentrations for inhibition of fermentation from Rothstein *et al.* (1948). In each case a reasonably straight line can be drawn through the points. The lines intersect the (U_T) axis very close to the origin. For this reason the value of K cannot be determined from the intercept with any accuracy. It is obviously low relative to values for (U_T), less than 1×10^{-6} (the value by chemical determination was $3\cdot 5 \times 10^{-7}$, shown in Table 1). On the other hand, the value for k can be readily calculated from the slopes of the lines to be about $7\cdot 6 \times 10^{-7}$ M for a yeast suspension containing 1 mg./yeast/ml. suspension. 1 mg. of yeast contains 1×10^7 cells; therefore, using Avogadro's number, each cell is predicted to contain about $4\cdot 6 \times 10^7$ binding sites directly involved in fermentation. The predicted value is exceedingly close to that found by actual experiment as shown in Fig. 4. As expected, from the assumption made in equation (4), the inhibition is proportional to the amount of uranium bound per cell, with essentially 100% inhibition when $4\cdot 6 \times 10^7$ molecules are taken up by each cell.

The $4\cdot 6 \times 10^7$ sites per cell involved in fermentation are the same ones whose properties were studied in regard to their mass-law behaviour (Table 1) and chemical identity (Fig. 2). However, these sites are not the only ones on the yeast cell capable of combining with uranium. The presence of uranium concentration greater than that required for complete inhibition of fermentation leads to a further uranium uptake, amounting

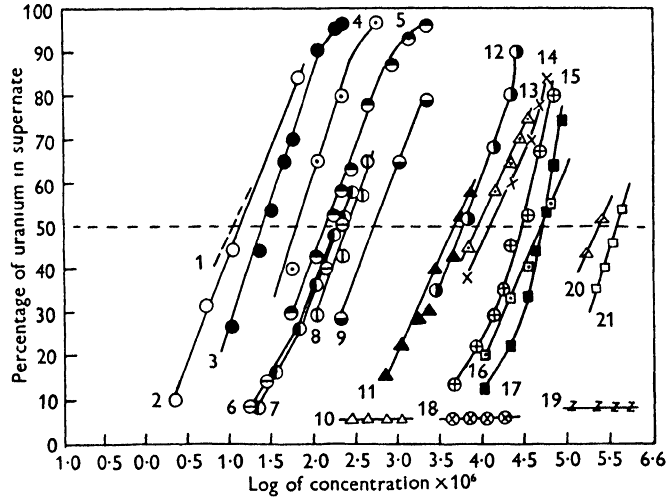

Fig. 3. The relationship beteeen yeast concentration and initial uranyl nitrate concentration at a fixed inhibition. (1) yeast; (2) metaphosphate polymer; (3) hexametaphosphate; (4) desoxyribonucleic acid; (5) pyrophosphate; (6) triphosphate; (7) ATP; (8) metaphosphate; (9) nucleic acid (tech.); (10) adenylic acid; (11) egg albumin; (12) serum albumin; (13) citrate; (14) HDP; (15) orthophosphate; (16) maleate; (17) glycerophosphate; (18) glucose-1-phosphate; (19) glucose; (20) acetate; (21) fructose.

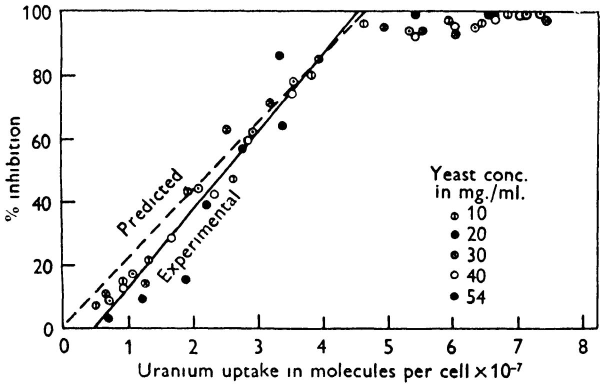

Fig. 4. The relationship beteeen uranium uptake by the cells and the inhibition of glucose metabolism.

to at least 3×10^7 sites per cell and perhaps more. The exact extent of this additional uranium binding cannot be readily determined for technical reasons. The complex formed under these conditions is relatively unstable, requiring high uranium concentrations to achieve saturation. The amount bound by the cells becomes vanishingly small compared to the amount of uranium added and falls within the limit of analytical error. The uranium-binding data indicate the existence of at least two species of uranium-binding loci in the cell, one more stable than the other. The 'stable sites' are involved in fermentation in a first-order relationship. The 'unstable sites' are not involved at all in fermentation.

In the case of respiration of glucose a more complicated situation exists. A concentration of uranium which gives essentially complete inhibition of fermentation results in only a 60% inhibition of respiration. The inhibition of the remainder of the respiration requires very much higher uranium concentration (Fig. 5). The uranium-binding properties of the cells are the same under aerobic as under anaerobic conditions, therefore it must be concluded that respiration involves more uranium-binding sites than does fermentation. The inhibition curve for respiration consists of two distinct phases. Sixty per cent of the respiration apparently passes through reactions held in common with fermentation involving cell-surface sites capable of forming a very stable complex with uranium. Forty per cent of the respiration passes through a non-fermentative pathway which involves surface sites which form a less stable complex with uranium. It seems more than a coincidence that a similar 60:40 relationship has been shown with urethane (Fisher & Stern, 1942). The uranium data are consistent with the concept that two respiratory pathways are functional in the cell, one which follows the fermentative scheme and one which does not. Perhaps the 'shunt pathway' through phosphogluconic acid is involved (Baldwin, 1952).

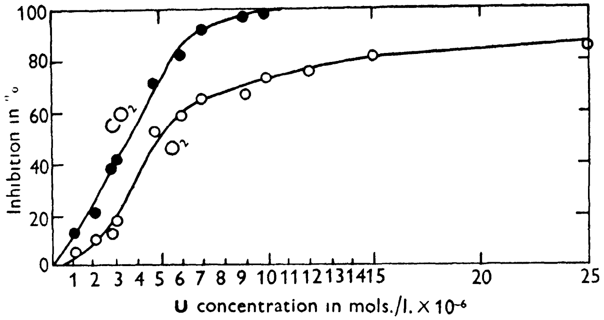

Fig. 5. The inhibition of fermentation and of respiration by uranium.

Differences in susceptibility to uranium have also been found in the case of fermentation of galactose as compared to glucose. Yeast does not normally ferment galactose, but pre-exposure of the cells to galactose plus glucose results in the appearance of 'adaptive fermentation' of galactose. The fermentation of galactose by 'adapted cells' is more sensitive, by a factor of about 2, to inhibition by uranium than is the fermentation of glucose by the same cells. Thus fewer surface sites are involved in galactose metabolism than in glucose metabolism (Rothstein, Meier & Hurwitz, 1951).

It has been pointed out previously that the membrane of the yeast cell is able to differentiate between glucose, fructose and mannose, as compared to galactose, sorbose and arabinose, the latter sugars being able to penetrate the cell membrane very slowly, if at all. On the basis of the uranium studies it must be concluded that other kinds of differentiation are also built into the membrane. First, the uptake of galactose in 'galactose-adapted' cells is more sensitive to uranium than is the uptake of glucose. Thus fewer surface sites are involved in the uptake of galactose than glucose. Secondly, glucose uptake under aerobic conditions involves more uranium-binding surface sites than does glucose uptake under anaerobic conditions. Furthermore, glucose uptake under aerobic conditions involves two kinds of sites, whereas glucose uptake under anaerobic conditions involves one. The organization of that part of the cell surface responsible for the uptake of sugars must be complex. It must contain mechanisms possessing an inherent specificity for certain sugars. In addition, it must contain two different systems for glucose uptake, one of which is operative only under aerobic condition.

VII. KINETIC STUDIES

In the presence of an inhibiting concentration of uranium, the inhibited reaction located at the cell surface determines the overall rate of metabolism. The cell-surface reaction can therefore be characterized in terms of kinetics and temperature effects if uranium is present. Yeast metabolism has been shown by Hopkins & Roberts (1935) and by Gottschalk (1944) to obey the Michaelis Menten equation

$$\frac{1}{V}=\frac{Km}{Vm}\frac{1}{S}+\frac{1}{Vm}, \tag{11}$$

where V is the rate of metabolism, S the substrate concentration, Vm the maximal rate of metabolism and Km the Michaelis constant. This equation is predicated on the existence of an enzyme substrate complex, the concentration of which determines the rate of the reaction. Thus

$$E+S\rightleftharpoons ES\rightarrow\text{product}. \tag{12}$$

Inherent in the equation is the concept of a limited number of enzyme sites. As the substrate concentration is increased, the rate of metabolism reaches a maximal value associated with the saturation of the enzyme sites with substrate. Higher concentrations of substrate cannot increase the rate of metabolism.

The Michaelis equation is tested by plotting $1/V$ against $1/S$. A straight line should obtain with an intercept equal to $1/Vm$ and a slope of Km/Vm. In Fig. 6, taken from Hurwitz & Rothstein (1951), it can be seen that fermentation and respiration give data consistent with the Michaelis equation not only in the absence of uranium, but also in those experiments in which an inhibiting concentration was present. It must therefore be concluded that an interaction between glucose and the cell occurs at the cell surface which involves a saturation phenomenon. In other words, glucose combines in some manner with some constituent in the cell surface which is present in limited concentration.

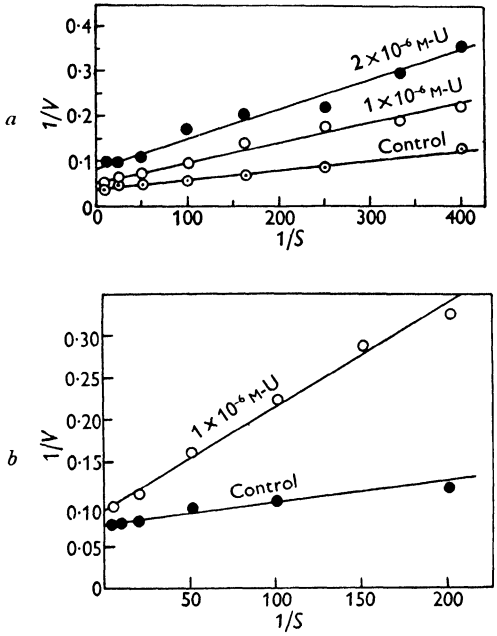

Fig. 6. *a*. Kinetics of inhibition of fermentation of glucose. *b*. Kinetics of inhibition of respiration of glucose.

It is possible further to characterize the inhibition in terms of the calculated values for Vm and Km (Baldwin, 1952). For example, in the case of the inhibition of fermentation by uranium in Fig. 6, the calculated value for Km is essentially the same for all three lines, $7{\cdot}2 \times 10^{-3}$, $8{\cdot}2 \times 10^{-3}$ and $8{\cdot}3 \times 10^{-3}$ for control, 1×10^{-6} M and 2×10^{-6} M uranium respectively. The Vm, however, shifts from 30 to 19 to 12 μl./mg./hr. Such behaviour is typical of a non-competitive inhibition. In the case of respiration, in the presence of uranium there is a fourfold shift in *Km* from $3{\cdot}8 \times 10^{-3}$ to $1{\cdot}9 \times 10^{-2}$, and a much smaller change in the *Vm* from 14 to 11 μl./mg./hr. Thus the kinetics of inhibition of respiration contains both competitive and non-competitive elements. Although the mechanism of inhibition of respiration is not obvious from the kinetic analysis, it is apparent that the inhibition kinetics of fermentation are different from the inhibition kinetics of respirations. This statement is based not only on the data of Fig. 6, but also on other kinetic data concerned with the effect of different uranium concentrations (Rothstein *et al.* 1951). The aerobic-anaerobic differences in kinetics are undoubtedly a reflexion of the phenomenon discussed previously. Fermentation proceeds through a reaction involving one kind of cell-surface site, whereas respiration proceeds by reactions involving two different kinds of cell-surface sites.

On the basis of the kinetic data certain suggestions can be made concerning the role of cell-surface reactions in sugar uptake. The agreement of the data with the Michaelis-Menten equation in the case of uranium-poisoned yeast suggests that glucose combines with a component in the cell membrane, the concentration of glucose-component complex determining the overall rate of metabolism. In fermentative glucose uptake, uranium does not prevent the formation of the glucose-component complex, but apparently prevents the breakdown of this complex into products, doing so by combining with necessary phosphate groups. An analysis of the respiratory glucose uptake is difficult because of the existence of two distinct mechanisms of glucose uptake.

VIII. TEMPERATURE EFFECTS

The temperature dependence of the uranium-inhibited reactions were characterized in terms of the Arrhenius equation

$$\log V = -\mu/2{\cdot}3RT + K, \tag{13}$$

where V is the rate of metabolism, μ the energy of activation, R the gas law constant and T the absolute temperature. In Fig. 7, log V is plotted against $1/T$. The control data can be conveniently represented by a pair of straight lines, the data is the presence of uranium by a single straight line. The values for μ calculated for the two lines of the control data were 21,000 cal./mol. for the lower segment and 13,000 cal./mol. for the upper, in essential agreement with the data of Stier (1933). The μ for the uranium curve was 22,000 cal./mol. The lines for the control and inhibited data tend to converge at higher temperatures. Thus elevation of the temperature from 20 to 30° C. decreased the inhibition from 66 to 29%.

The value of μ of 22,000 cal./mol. is inconsistent with any mechanism involving a free diffusion of glucose through the membrane in the aqueous phase. It does not, however, exclude the concept of activated transport through a lipid membrane as postulated by Danielli (1943), although it is doubtful, in view of the low solubility of sugars in lipids, that movement through a lipid phase occurs to any extent in sugar uptake. Such a mechanism could not account for the selectivity of the membrane in regard to various sugars, for the uranium effects or for the kinetics. Thus the high-temperature coefficient is undoubtedly the characteristic of a chemical reaction between glucose and a cell-surface constituent.

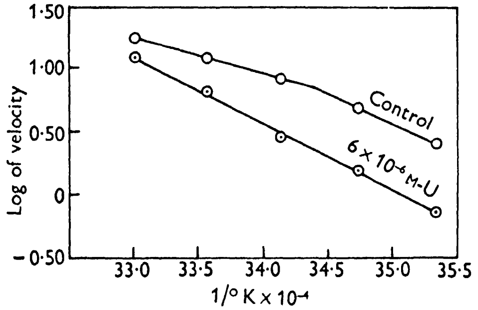

Fig. 7. Temperature characteristics of respiration of glucose in the presence and absence of uranium.

IX. THE EFFECT OF EXTRACELLULAR pH ON THE RATE OF FERMENTATION

It has been suggested that the initial phosphorylation reactions in sugar metabolism are mediated by enzymes located on the surface of the cell. Such enzymes should be susceptible to the influence of extracellular pH. Yet the reports in the literature indicate that fermentation is remarkably independent of pH over a wide range (Euler & Heintz, 1919; Hagglund & Augustson, 1925), a situation which is seemingly incompatible with the hypothesis. The explanation of this contradiction lies in the fact that the previous studies were made using potassium phosphate or potassium citrate buffers. As will be shown, potassium counteracts the depressant effect of hydrogen ion on fermentation, with the result that in the presence of high potassium concentrations, the rate of fermentation is apparently independent of pH over a wide range. However, if inert buffer systems are used, fermentation is found to be markedly dependent on pH (Rothstein & Demis, 1953*b*).

Inert buffers were found for the pH range 2·0–6·0 (triethylamine(TEA)-succinate-tartrate) and 8·0–10 (tris-hydroxymethylaminomethane or

THAM). In the range 6·5–7·5 no adequate buffers were found, therefore, constant pH was maintained manually by addition, drop-wise, of TEA. Manometric determinations of oxygen consumption and carbon dioxide production were feasible in the range 2·0–6·0 and 8·0–10, with results which agreed closely with the rates of sugar consumption. In the range of pH 6·5–7·5, metabolism was measured only in terms of glucose consumption.

No pronounced differences were observed between the effects of pH on respiration as compared to those on fermentation. However, because there were marked effects of pH on the end-products of fermentation, the data presented here will concern this mode of metabolism.

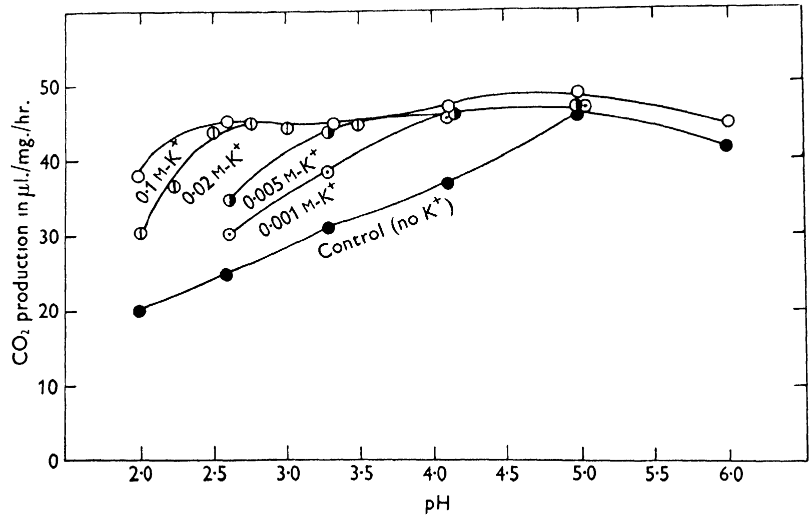

Fig. 8. The effect of pH on the rate of fermentation and on the end products of fermentation.

The pH activity curve for anaerobic glucose uptake by living cells is a biphasic curve, with optima at pH 5·5 and 8·5. Yeast can ferment glucose over the pH range of 1·7–10·5, yet the average internal pH of the cytoplasm remains constant. For example, Conway & Downey (1950*b*) have shown that during active fermentation in the presence of potassium with the external pH dropping rapidly to a value below 2·0, the internal pH of the cell becomes slightly more alkaline. Using Conway's freezing and thawing procedure it was found that yeast fermenting in media maintained at pH's ranging from 2·0 to 10·0 has a relatively constant internal pH in the range 6·2–6·4. Thus the rather dramatic effects of extracellular pH shown in Fig. 9 must be characteristic of reactions occurring in the periphery of the cell, exposed to the varying extracellular pH rather than to the constant internal pH. The fact that the pH curve is biphasic is not entirely surprising.

It would be difficult to imagine any single enzyme reaction which could show adequate activity over the wide pH range (1·7–10·5) over which cells show fermentative capacity. In general, enzymes have a considerably more restricted zone of activity in regard to pH, encompassing perhaps 4–5 pH units. On the basis that surface enzyme activity is necessary, it would seem reasonable that the ability of yeast to take up glucose over an extended pH range lies in the possession of two different surface enzymes, each responsible for a portion of the pH range. Other evidence of the existence of two surface enzymes has been found. At pH 8·5 mannose is respired 55% as rapidly as glucose, but at pH 3·5 mannose is respired 92% as rapidly as glucose. At pH 8·5 glucose uptake is inhibited by calcium, whereas at pH 2·0–6·0, glucose uptake is markedly increased by calcium. In fact at pH 6 glucose uptake can be made almost entirely dependent on the presence of calcium or other bivalent ions (see § XI).

There is some likelihood that the surface enzyme in alkaline fermentation is the hexokinase crystallized by Kunitz & McDonald (1946) and Berger, Slein, Colowick & Cori (1946). At least it has many similar properties. It has a pH optimum on the alkaline side, it is only half as active toward mannose as towards glucose, and it is inhibited by calcium.

The crystalline hexokinase could not account for fermentation in acid solutions. It has a low activity at pH 5·0, less than 10% of that at pH 8·0. It has no measurable activity below pH 4·0 (Hurwitz, 1953). Nevertheless, in cell-free preparations considerable fermentative activity is found below pH 4·0. It seems possible that a second hexokinase, with an acidic pH optimum, is responsible for the fermentation at low pH in both cell-free preparations and in living cells. Proof of its existence, of course, will only be established by its isolation and characterization.

In addition to the effects of extracellular pH on the rates of fermentation, there are some rather dramatic alterations in the end-products of fermentation. It has been known for some time that under alkaline conditions there is an increased production of glycerol by fermenting yeast. Thus Neuberg (Baldwin, 1952) classifies his third method of fermentation as the production of 2 mol. of glycerol, 1 mol. of alcohol and 1 mol. of acetic acid. Recently, Neish & Blackwood (1951) showed that as the pH is increased to 8·2, the glycerol and acetate production during fermentation increase relative to alcohol production. Rothstein & Demis (1953*b*) have done carbon balance studies concurrently with the studies of the effects of pH on rates of fermentation (Fig. 7). In the pH range 2·0–6·0 the end-products are largely alcohol (35%), carbon dioxide (20%), and glycogen (35%) with only small amounts of glycerol and acetate. In the pH range 8·0–10·0, the alcohol and carbon dioxide remain about the same, but there is almost no net glycogen synthesis. In place of glycogen, there is a marked increase in glycerol production and also some increase in acetate. Sussman, Spiegelman & Reiner (1947) had previously noted that assimilation of glucose was decreased at alkaline pH. Wiggins, Mann, Trevelyan & Harrison (1952), on the other hand, found no decrease in glycogen synthesis at pH 8·5. The basis for the discrepant results does not seem obvious at the present time, unless it is due to differences between strains of yeast.

The pH at which the change-over in end-products occurs corresponds to the dip between the two optima in the pH curve for sugar uptake (Fig. 7). The pH curve for glycerol production corresponds almost exactly with the alkaline phase of the pH curve for sugar uptake. In view of the fact that the internal pH remains constant it must be concluded that reactions occur at the cell surface, susceptible to the external pH, which not only determine the rate of sugar uptake, but also determine the nature of the end-productions of fermentation. The surface mechanism for sugar uptake is not simple in that it merely delivers glucose or a product of glucose metabolism into the interior of the cell for conversion there into end-products. Instead, it is complex, containing within itself capacities which determine the nature of the end-products.

X. THE EFFECT OF MONOVALENT CATIONS ON CELL-SURFACE REACTIONS

Potassium is present in relatively high concentrations in most cells. In the yeast cell its concentration in the cytoplasm is of the order of 0·1 M. Potassium is related to carbohydrate metabolism in yeast in at least two ways. In the first place potassium can stimulate the rates of fermentation, respiration and glucose consumption (Lasnitzki & Szorenyi, 1935; Farmer & Jones, 1942; Rothstein & Enns, 1946). In the second place, during metabolism of sugar, potassium is taken up by yeast cells (Pulver & Verzar, 1940) in a reaction which involves an exchange for hydrogen ions produced by cell metabolism (Rothstein & Enns, 1946; Conway & O'Malley, 1946). Conway has studied the K^+–H^+ exchange in some detail and has presented a general theory of the mechanism of acid secretion (Conway, 1953).

The relationship between the two K^+ phenomena has been investigated by Rothstein & Demis (1953*a*). Several possibilities seemed worthy of consideration. Potassium might increase the rate of fermentation by stimulating reactions occurring on the surface of the cell. Or, because potassium is taken up by the cell in exchange for hydrogen ions, the stimulation of metabolism might be associated either with the increased intracellular potassium content, or with the increased acid secretion.

The stimulation of sugar uptake by potassium is dependent on two factors, the potassium concentration and the hydrogen-ion concentration. There are two zones of pH in which potassium has a substantial influence, 2·0–4·0 and 5·5–7·5, corresponding to the depressed portions of the pH-activity curve of fermentation (Fig. 7). Because the K stimulations in the two zones seem to be mediated by different mechanisms, they will be discussed separately.

In the lower pH range the magnitude of the effect increases as the pH is decreased, but higher concentrations of potassium are required to invoke the maximal effect. The rate of metabolism in the absence of potassium decreases from a maximum at pH 5·0 to 43 % of normal at pH 2·0, but the maximal rate of metabolism in the presence of potassium is essentially the same at all values of pH (Fig. 8). It appears, therefore, that hydrogen ion depresses the rate of fermentation and that the appropriate concentration of potassium can counteract the inhibiting effect. The higher the concentration of hydrogen ion, the higher must be the potassium-ion concentration to achieve reversal. The interaction of potassium and hydrogen ions apparently takes the form of a direct competition for the same loci because there is a fixed relationship between the concentrations of the two ions. Thus with any given hydrogen-ion concentration above 1×10^{-5} M, a

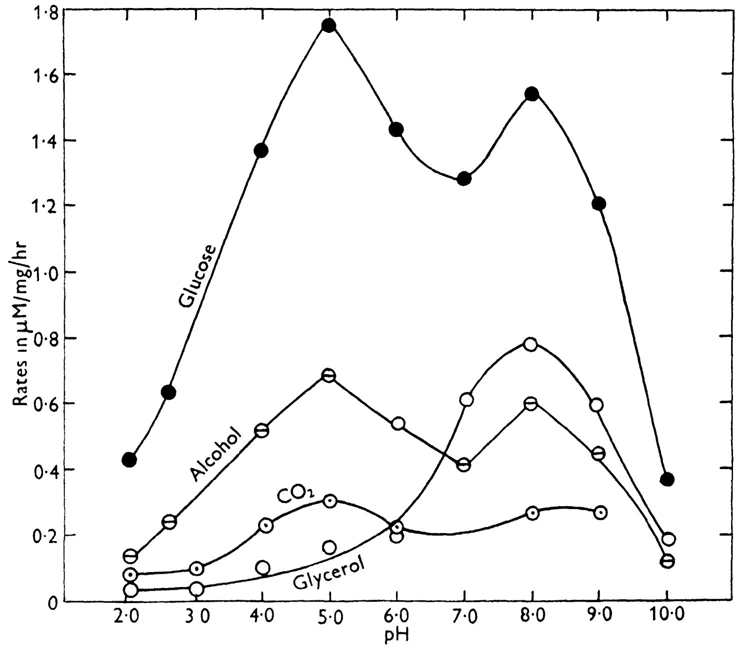

Fig. 9. The stimulation and fermentation by potassium as influenced by pH.

maximal rate of metabolism can be obtained with a ratio of K^+ to H^+ of approximately 10 to 1.

Under certain conditions, the effect of potassium on the rate of fermentation is considerably more dramatic. In the experiments described in the preceding paragraph, the cells were starved with aeration for several hours before they were used. If the cells are starved for a longer period of time, of the order of 24 hr., they lose a considerable amount of potassium. Such cells have a very low rate of metabolism at pH 2·0, but the addition of 0·2 M-KCl will instantly return the rate to almost maximal value, a stimulation of over 400%.

Potassium ion exerts its effect predominantly on fermentation rather than respiration. For example, at pH 2·7 the addition of 0·02 M-KCl to a yeast suspension resulted in an increased rate of sugar consumption amounting to 83% under anaerobic conditions compared to 69% under aerobic conditions. However, the increased rate of uptake under aerobic conditions is largely due to an increase in the rate of aerobic fermentation. Thus, if the potassium effect is measured in terms of carbon dioxide production and oxygen consumption, then the stimulation of fermentation is 84%, and the stimulation of respiration only 25%. There is an increase in the R.Q. from 1·7 to 2·5, and aerobic fermentation is increased 146%. It can be calculated that about 90% of the increase in the rate of aerobic glucose consumption is due to the increased aerobic fermentation. Respiration of lactate, pyruvate and alcohol is stimulated to about the same extent as is the respiration of glucose, about 20%.

Other cations were tested for their effect on fermentation at pH 2·7. In each case the concentration was 0·02 M. Potassium had by far the greatest effect, 83%, followed in order by rubidium 40%, calcium, magnesium and manganese about 20–25%, sodium 15–20%, ammonium, lithium and caesium 10%. None of the other ions reduced the stimulating action of potassium.

The stimulating action of potassium in the pH range 5·5–7·5 can be very dramatic if the cells are starved for a few hours and then thoroughly washed with distilled water. For example, at pH 6·0, the stimulation of fermentation of glucose is over 100%. If the cell suspension is first treated with a TEA-cation exchange resin (Dowex 50), the potassium stimulation is greater than 700%. At pH 6·0, the rate of fermentation is normally considerably lower than at pH 5·0 or 8·5 (Fig. 7). Treatment with resin reduces the rate at pH 6·0 to a very low level but has much less effect on the rates at pH 5·0 and 8·5. Thus the cells are particularly dependent on potassium at pH 6·0. The presence of potassium returns the rate to the same level found at pH 5·0 or 8·5, without added potassium.

The potassium dependence of fermentation at pH 6·0 does not seem to be the same as the potassium dependence at low pH discussed previously. In the first place, NH_4^+ can displace K^+ at pH 6·0 but not at low pH. The stimulation of fermentation by NH_4^+ was first noted by Zeller (1926) and by Smythe (1939). The effect seems to be due to NH_4^+ rather than undissociated NH_4OH. In the second place the stimulation at pH 6·0 is associated with an increased glycerol fermentation, whereas that at pH 2·0–4·0 leads to an increased alcoholic fermentation. Thus the stimulation at pH 6·0 seems to be primarily of the pathway of metabolism associated with the alkaline phase of the pH curve, with its optimum at pH 8·5.

Yeast, during metabolism of sugars, secretes acid with a resultant decrease in the pH of the medium. In an unbuffered medium with no salts present, the pH will drop slowly to 3·5, due to succinic acid secretion. In the presence of 0·02 M-KCl, the pH drops rapidly to a minimum of 2·6, a consequence of the exchange of K^+ for H^+. If the concentration of potassium is increased the minimum pH is decreased. Thus Conway & O'Malley (1946) found that with 0·2 M-KCl, the pH dropped to values as low as 1·7 Other ions such as rubidium, sodium and lithium also are exchanged for H^+, but at a much slower rate (Conway, 1953; Rothstein & Demis, 1953*a*).

Is the K^+ stimulation which is discussed in the previous section related to the K^+–H^+ exchange? The exchange reaction is rapid at higher pH's, but with 0·02 M-KCl approaches zero at pH 2·6–2·7 and is actually negative (in the reverse direction) below pH 2·6. On the other hand, the stimulation, of metabolism with the same potassium concentration is greatest at pH 2·0. decreasing as the pH is raised to 5·0. Thus the stimulating action of potassium is observed, whether there is a net exchange of cellular H^+ for K^+ from the medium, no net exchange, or a net exchange in the opposite direction. The stimulation of metabolism by potassium is therefore not directly associated either with the secretion of hydrogen ion by the cell, or with the uptake of potassium by the cell.

Further light is thrown on the potassium effect by studies of cell-free yeast preparation similar to that prepared by Meyerhof & Kaplan (1951). The cells are slowly dried at room temperature, treated with acetone, then lyophylized and pulverized (Rothstein & Demis, 1953*b*). Such a dried yeast is cell-free, and it will ferment glucose rapidly with no lag period. The preparation was treated with cation resin previously neutralized with TEA. Thereby all of the normal cations of the yeast are replaced by TEA. A mixture of bivalent ions is added to the yeast preparation in a TEA-succinate-tartrate buffer. Such a preparation will only ferment glucose if potassium is added. It has a narrower pH-dependence curve than live yeast. In addition to its absolute dependence on potassium, such a preparation shows a H^+–K^+ relationship similar to that of live yeast. As the pH is reduced the rate of metabolism is reduced, but can be counteracted to some extent by an increased potassium concentration. In such a preparation the cell membrane is not intact. It leaks proteins and other cytoplasmic constituents such as organic phosphate and potassium. There is no impediment to movement of K^+, H^+ or glucose to the enzyme sites and yet the inhibiting effect of H^+ and counteracting effect of K^+ can be demonstrated. Thus the effect of K^+ on metabolism can probably be attributed to its action on fermentation enzymes rather than to its effect on acid secretion or on some permeability property of the membrane.

The normal content of potassium in yeast is of the order of 0·1 M. In view of the fact that concentration in the medium as low as 0·0003 M can evoke a metabolic effect, and in view of the fact (see preceding section) that the stimulation can occur under conditions in which there is no net uptake of potassium, it would seem that the intracellular potassium is not involved in the observed phenomenon. These observations were confirmed experimentally by manipulating the intracellular concentration of potassium. High-potassium yeast was prepared by pre-exposing cells to glucose plus potassium in citrate buffer at pH 4·5. These cells took up potassium equivalent to 0·05 M/l. of cells. Low-potassium yeast was prepared by starving for 24 hr. They lost 0·03 M of potassium per litre of cells. The three types of cells, low potassium, normal and high potassium, had cellular potassium concentrations of 0·07, 0·1 and 0·15 M, a twofold range. The maximal rates of fermentation induced by extracellular potassium (0·02 M) was the same in each case (40–41 μl./mg./hr.) (Rothstein & Demis, 1953*a*), even though no additional K^+ was taken up during the course of the experiment. It must be concluded therefore that the fermentation reactions influenced by potassium are peripherally located in the cell, where they are influenced by extracellular rather than intracellular potassium.

XI. THE EFFECTS OF THE BIVALENT IONS ON SURFACE REACTIONS

Uranyl ion inhibits sugar uptake by combining with polyphosphates on the cell surface, substances which may be involved directly in phosphorylation reactions. Phosphorylation reactions in general require the presence of bivalent ions for maximal activity, particularly magnesium and sometimes manganese. An attractive hypothesis for explaining the uranium effects involves the displacement of magnesium and manganese ions by a competitive effect from phosphorylation reactions requiring these ions.

The existence of competition between various bivalent ions and uranyl ion was shown in two ways. In the first studies (Rothstein & Meier, 1951), yeast cells were equilibrated with uranium resulting in the binding of 95% of this ion. On the addition of other bivalent ions, some of the uranium was displaced from the cells by competition and appeared in the medium. Magnesium, calcium, barium and zinc showed competitive effects. Sodium and potassium did not. However, the cell-surface sites had a far greater affinity for uranyl ion than for any of the other ions tested. For example, the affinity for uranyl ion is of the order of several thousand times that for magnesium or calcium. In the second technique, displacement of uranium from the surface sites was not measured chemically, but instead, in terms of a reduction in the inhibiting action of uranium. Magnesium, calcium and manganese were tested and all were able to reduce the inhibiting action of uranium (Hurwitz, 1953; Rothstein & Hayes, 1953). This experiment indicates not only that uranyl ion can be displaced by the bivalent ions tested, but also that surface sites combined with magnesium, calcium or manganese are *metabolically active*. The question remains as to whether the cell *tolerates* or *requires* that the surface sites be present in the form of a magnesium, calcium or manganese complex for sugar uptake to proceed.

The relationship of the binding of bivalent cations by the cell surface to the uptake of glucose has been investigated by Rothstein & Hayes (1953). Isotope studies with ^{55}Mn and ^{45}Ca reveal that the cell can reversibly bind a fixed number of cations. All of the bivalent ions tested, including Mn^{++}, Ca^{++}, Mg^{++}, Ba^{++}, Co^{++}, Zn^{++}, Hg^{++}, Cu^{++} and UO_2^{++} compete for the binding sites, but of these ions, UO_2^{++} has by far the greatest affinity. The equilibrium between the ions and the cells is attained very rapidly (less than 3 min., the fastest time that could be measured) and does not alter appreciably during the next hour. If the cells are equilibrated with ^{55}Mn or ^{35}Ca and are then resuspended in solutions of non-isotopic Mn^{++} or Ca^{++}, there is an immediate back exchange of the labelled ions, with the attainment of the same equilibrium point, whether the experiment is carried out with unlabelled cells in solutions containing the labelled ions or with labelled cells suspended in solutions containing the unlabelled ions. For example, in Table 2, in the experiments with no added phosphate (column A), in the presence of $0{\cdot}33 \times 10^{-4}$ M-Mn^{++}, 66% of the ^{55}Mn is taken up within 3 min. and no further uptake occurs for 1 hr. In the presence of $6{\cdot}0 \times 10^{-4}$ M-Mn^{++} (column C), only 44% is taken up, because of the greater dilution of ^{55}Mn with unlabelled Mn^{++}. Again the equilibration is complete in 3 min. Another sample of yeast first equilibrated with $0{\cdot}33 \times 10^{-4}$ M-Mn^{++} also shows 66% binding of ^{55}Mn. After 15 min. the Mn^{++} concentration is increased to $6{\cdot}0 \times 10^{-4}$, and within a few minutes some of the ^{55}Mn is displaced from the cell, and the new equilibrium is the same as that which would have resulted had the higher concentration of Mn^{++} been present initially (column B). The same equilibrium point is achieved in either direction.

Table 2. *The influence of phosphate on the uptake of* ^{55}Mn *in the presence of different concentrations of* Mn^{++}

Time (min.)	Percentage ^{55}Mn uptake				
	No phosphate			Plus phosphate	
	A	B	C	D	E
3	66	64	44	48	—
15	65	66	45	85	—
20	—	47	—	95	—
45	65	48	46	100	100
60	65	46	45	100	98
90	—	—	—	—	—
120	—	—	—	100	97
Conc. of Mn^{++} added	0·33 × 10^{-4} M	0·53 × 10^{-4} M until 15 min. then increased to 0·33 × 10^{-4} M	6·0 × 10^{-4} M	0·33 × 10^{-4} M	0·35 × 10^{-4} M until 45 min. then increased to 6·0 × 10^{-4} M

Yeast concentration 200 mg./ml., pH 5·5, glucose 0·2 M, potassium chloride 0·02 M, TEA-succinate-tartrate buffer. A trace amount of ^{55}Mn is added in each experiment, sufficient to give 350 counts per ml. of the medium.

The characteristics of the binding of bivalent cations by the yeast cell are similar to those described for cation exchange resins. The mass-law derivations that have been applied to the exchange resins apply just as well to yeast. The concentration of binding sites calculated from mass-law measurements of ^{55}Mn binding is approximately 1×10^{-3} M/l. of cells, a figure which is in good agreement with that determined by studies of uranium binding. The dissociation constant is of the order of 1×10^{-3} for Mn^{++}, Mg^{++} and Ca^{++}, a figure which is only approximate because of the long extrapolation involved. On this basis these cations form complexes less stable than that of uranium by a factor of about 5000, a figure which agrees with the relative stabilities calculated by direct competition.

There is considerable evidence that the binding and exchange of bivalent ions as described in the preceding paragraphs takes place only at the surface or periphery of the cell. For example, the existence of competition of UO_2^{++}, Ca^{++}, Mg^{++} and Mn^{++}, and the almost exact correspondence between the number of uranium binding sites and Mn^{++}, Ca^{++} and Mg^{++} binding sites, indicates that the binding sites for all of these cations are identical. It has already been shown that the uranium-binding sites are located on the surface of the cell, therefore the Mn^{++}, Ca^{++} and Mg^{++}

binding sites must also be located on the surface of the cell. In addition, evidence independent of the uranium competition can be cited. First, the extremely rapid equilibration of the cell with the bivalent ions of the medium argues for a surface phenomenon. Complete equilibration of the contents of the cytoplasm would presumably take more than 3 min. Secondly, the maximal binding of the bivalent ions by the cells is about 1×10^{-3} M/l. of cells. Since the bivalent cation content of the total cytoplasm in the same cells is about 40×10^{-3} M/l. of cells, only 2·5 % of the bivalent cations of the cell can participate in the exchange for ions in the medium. Thus the bulk of the bivalent cations of the cytoplasm are not in communication with the environment. It is therefore suggested that those ion-binding sites located on the cell surface can readily equilibrate with the medium. This concept is similar to that put forth by Mazia (1940) and Lansing (1942).

The absence of exchange between bivalent cations of the interior of the cell and those of the medium has also been demonstrated by labelling the interior compartment with ^{55}Mn or ^{35}Ca (Goodman & Rothstein, 1953). Labelling of the cytoplasm was accomplished by setting up conditions whereby the bivalent cations were actively transported from the medium into the cell. Resting cells, as already indicated, do not actively take up the bivalent cations of the medium; they simply equilibrate in a manner dictated by mass-law considerations. The addition of glucose and the resultant appearance of exogenous metabolism, either aerobic or anaerobic, does not alter the situation. However, if phosphate is added together with the glucose, then the bivalent ions are actively transported into the cell against the concentration gradient. In Table 2, column D, it can be seen that in the presence of phosphate all of the measurable ^{55}Mn has been taken up by the cell. The Mn^{++} taken up in this manner is no longer in equilibrium with the environment for it cannot be washed out, nor can it be exchanged back when the Mn^{++} concentration of the medium is increased (Table 2, column E). The amount of Mn^{++} that can be transported into the cell in the presence of phosphate can be considerable, amounting to 0·02 M/l. of cells. This represents a 50 % increase in the bivalent ion content of the cell and 20 times the maximal reversible binding of Mn^{++} in the absence of phosphate. The detailed mechanism of the bivalent cation transport is beyond the scope of the present discussion, but it can be briefly stated that it depends on the active transport of the phosphate, which carries the cation into the cell in the form of a soluble complex.

The studies of bivalent ion uptake support the conclusion that the reversible binding in the absence of phosphate is indeed an equilibration only with sites in the periphery of the cell and not with those in its interior.

Once carried into the interior of the cell, a bivalent cation is no longer exchangeable with environmental ions. How does the binding of various bivalent ions on the surface of the cell influence the ability of the cell to take up glucose? The initial studies indicated that Mn^{++}, Mg^{++} and Ca^{++} could each stimulate the uptake of glucose about 20–30 %. Attempts to make the cells more dependent on environmental ions were carried out by deionizing by prolonged washing and starving of the cells, and finally, more successfully, by treating the cells with cation exchange resins in the form of the triethylamine (TEA) salt. By these means it was possible to reduce considerably the ability of the cell to take up glucose. The rate of glucose uptake could then be returned to normal by adding back the various ions. The predominant effects were obtained with K^+ (see preceding section), but the metabolism was also remarkably dependent on the presence of the bivalent ions, particularly at certain values of extracellular pH. Sample data are given in Table 3. At pH 3·5, each of the ions stimulates 20–25 %, but at pH 6·0 they stimulate 100 %. If the cells are pretreated with TEA resin, the control rates at pH 6·0 can be reduced almost to zero, and the metabolism is then completely dependent on the presence of extracellular ions.

Table 3. *Effect of bivalent ions on fermentation of glucose*

	pH of the medium		
	3·5	6·0	8·5
Control	25	14	39
TEA	—	14	—
Ca	32	31	33
Mg	30	27	38
Mn	30	28	38

Data are in μl. of CO_2/mg./hr. Ions are 0·003 M.

The effects of bivalent ions are not simple. The dependence of glucose uptake on these ions varies considerably with extracellular pH. At pH values below 6·0, Mg^{++}, Mn^{++} and Ca^{++} all stimulate, especially at pH 5·5–6·0. At pH 8·5, none of them stimulate, but Ca^{++}, in contrast to Mg^{++} and Mn^{++}, inhibits about 50 % at 0·05 M. Regardless of complications it can be concluded that the surface sites involved in the binding of bivalent ions are also involved in some manner in the uptake of glucose. In fact, under certain conditions the uptake of glucose proceeds at very low rates unless bivalent ions are present.

XII. PHOSPHATE UPTAKE AND PHOSPHATASES

Kamen & Spiegelman (1948) have suggested that uptake of phosphate by micro-organisms involves an active transport system. In the case of yeast cells, there is clear-cut evidence in this regard. Resting cells do not exchange phosphate, lose phosphate or take up phosphate at any appreciable rate (Hevesy, 1948). In the presence of glucose, however, there is a rapid uptake of phosphate by the cell and incorporation into metaphosphate compounds (Wiame, 1949). In the presence of low concentrations of azide (Spiegelman, Kamen & Sussman 1948) or dinitrophenol (Hotchkiss, 1944), the phosphate uptake is repressed, even though the rate of fermentation is not diminished. If these poisons in low concentrations act by 'uncoupling' oxidative steps from phosphorylation, then it is apparent that phosphate uptake in the intact cell is not only metabolism connected, but is directly associated with phosphorylation mechanisms.

The uptake of phosphate during sugar metabolism does not seem to be an inward diffusion in response to a concentration gradient associated with a reduced internal phosphate concentration consequent to phosphorylation processes, a mechanism aptly described by Rosenberg & Wilbrandt (1952) as 'trapping'. In the first place there is almost no exchange of phosphate between the cells and the medium either in the absence of glucose (Hevesy, 1948) or in the presence of glucose, during the rapid uptake of phosphate, as shown by the constancy of the specific activity of the extracellular phosphate using ^{32}P (Rothstein & Meier, 1949). This indicates that the process of phosphate uptake involves a movement only in the inward direction, a phenomenon incompatible with the concept of an inward movement of phosphate in response to a concentration gradient. In the second place, orthophosphate moves into the cell against the concentration gradient. Data taken from Rothstein & Meier (1949) and Schmidt, Hecht & Thanhauser (1949) indicate an appreciable uptake of phosphate when the extracellular concentration is as low as 0·0004 M. The intracellular orthophosphate concentration in these experiments was of the order of 0·01–0·02 M, or 25 times as high.

The evidence presented above suggests that phosphate is taken up by a mechanism involving incorporation of phosphate into phosphate compounds at the cell surface. The storage form of phosphate in the cell is metaphosphate (Wiame, 1949; Schmidt *et al.* 1949). However, in view of the high energy content of the phosphate linkages in metaphosphates it seems unlikely that phosphate is directly incorporated into such compounds. A more likely explanation is the uptake of orthophosphate by phosphoglyceraldehyde, an essential reaction in the fermentative scheme (Baldwin, 1952).

Phosphatases have often been thought to play a role in absorption of sugars (Danielli, 1952). In view of the fact that phosphatases have been localized at the surface of the yeast cell, their function has been investigated (Rothstein & Meier, 1949). In the presence of low concentrations of molybdate or tungstate, the phosphatases were completely inhibited, but there was no measurable effect either on phosphate uptake or on sugar uptake. Thus these particular phosphatases play no role in either process.

XIII. WHAT IS THE CELL SURFACE?

In this paper, reactions are classified as 'cell-surface' reactions, if they are directly influenced by the external environment rather than by the internal environment of the cell, provided that the reaction is not associated with a secreted enzyme, and provided that there is no alteration of the internal environment that could account for the effect. The internal environment is defined in terms of constituents which can be extracted from the cell by such techniques as freezing and thawing, by drying, by extracting, etc. These constituents, such as K^+, H^+ and orthophosphate, are presumed to be distributed in the cytoplasmic water. On this basis the cell surface is defined in terms of a *barrier* which separates the zone of influence of the inside environment from that of the outside environment. Cell-surface reactions occur on or outside of the barrier.

On the basis of the data on living yeast cells presented in this section, it is not possible to make definitive statements concerning the structure of the barrier. However, other experiments with a cell-free, fermenting system throw some light on the problem. The fermentative enzymes of yeast are soluble proteins. Many soluble 'zymase' preparations have been prepared which can ferment glucose. Yet a cell-free preparation has been prepared as described in § X, which contains over 80 % of the fermentative capacity of the cell in an insoluble residue, even when suspended in an ionic environment similar to that of the yeast cytoplasm (Rothstein & Demis, 1953*b*). It produces alcohol, CO_2 and glycogen, but has almost no ability to respire. It is therefore suggested that in the intact cell the fermentative enzymes are also retained in an insoluble structure. Furthermore, the structure possesses some kind of a permeability barrier. When it ferments glucose in the presence of inorganic phosphate, it takes up the phosphate and incorporates it into phosphorylated intermediates of metabolism which are retained within the structure. These do not appear in the medium unless the structure is treated with 5 % trichloroacetic acid, in which case they can be extracted. On the other hand, although the structure can ferment glucose, producing phosphorylated intermediates, the latter compounds when added to the medium cannot be fermented. Thus there is an insoluble residue of the cell, which contains the bulk of the fermentative activity of the cell and which has a permeability barrier to the diffusion of phosphate esters in either direction. This barrier apparently has no relationship to the general permeability barrier of the cell, which is destroyed by the method of preparation, with the resultant leakage of most of the soluble cytoplasmic constituents such as potassium and inorganic phosphate, as well as proteins. For convenience the cell-free, insoluble, fermenting structure will be called the 'glycosome'.

The evidence previously presented concerning the actions of environmental factors on glucose and phosphate uptake have been interpreted on the basis that initial steps in fermentation are located in the periphery of the cell. The evidence that the bulk of the fermentative activity of the cell is contained in a structural element, the 'glycosome', suggests that this element must also be located in the periphery of the cell, perhaps as a shell which constitutes all or part of the cortex of the cell. Additional support for this view is found in the similarities of the properties of the 'glycosome' and the surface of the intact cell. Both are impermeable to phosphorylated intermediates of metabolism. Both can take up and esterify inorganic phosphate. Both are inhibited in regard to glucose uptake by low pH, with a reversal of this effect by K^+.

The 'glycosome' differs from the intact cell in that in addition to the K^+–H^+ ion effects, its fermentation has an absolute dependence on the presence of K^+ even at the pH optimum, whereas the living cell does not. Presumably the structure of the 'glycosome' is such that its outer surface, that which is exposed to the environment, contains reactions in sugar metabolism which are not absolutely dependent on K^+, but which show inhibition by H^+ and reversal by K^+. The inner surface and perhaps the interior of the 'glycosome' contain reactions with an absolute dependence on K^+, but this does not show in the living cell because the inner surface is always exposed to the high K^+ content of the cytoplasm.

XIV. CONCLUSIONS

A number of observations have been made concerning interactions taking place at the surface of the yeast cell, interactions which are responsible for the uptake of sugars from the extracellular environment. The pertinent facts can be listed as follows:

(1) The cell surface is highly selective towards various sugars.

(2) There is more than one surface mechanism by which glucose can be taken up, one operative only under aerobic conditions.

(3) The surface reactions show properties typical of enzyme reactions in regard to kinetics and temperature effects.

(4) Polyphosphates and bivalent ions such as magnesium and manganese are involved directly in sugar uptake.

(5) Orthophosphate is esterified at the cell membrane in connexion with sugar uptake.

(6) The pH activity curve for sugar uptake is biphasic, covering a range from less than 2·0 to greater than 10·0, but the interior pH of the cell remains constant.

(7) Substances acting at the cell surface such as H^+ and NH_4^+ can alter the sugar uptake not only quantitatively, but qualitatively as well, in terms of an alteration in the end-products of fermentation.

(8) Extracellular potassium can markedly stimulate fermentation of living cells, whereas H^+ inhibits. Similar effects have been shown in a cell-free system in which the permeability barrier is destroyed.

(9) A cell-free preparation can ferment glucose, but not sugar phosphates, indicating the presence of an intact structural unit impermeable to sugar phosphates but permeable to glucose. Only on autolysis are the enzymes for metabolizing sugar phosphates liberated.

It is obvious that a mechanism of considerable complexity is located at the outer boundary of the cell, a mechanism with the function of absorbing sugars. Not only is specificity for particular sugars built into this mechanism, but in addition specific differences related to the nature of metabolism. Thus glucose taken up under aerobic conditions is moved through the cell surface by two mechanisms, one of which is not operative in glucose uptake under anaerobic conditions even though the single anaerobic mechanism has a capacity for sugar uptake which is almost twice that accomplished by both aerobic mechanisms.

K^+ and NH_4^+, which influence the fermentative uptake of sugars by acting on the cell-surface mechanisms, not only alter the sugar uptake in a quantitative manner, but exert qualitative effects on the course of metabolism in terms of the nature of the end products. It seems, therefore, that the surface mechanism is not simply a means for moving sugar into the interior of the cell where it can then pass through a series of reactions controlled by the cytoplasmic environment, but rather that the reactions at the cell surface must proceed to the point where the determination of the end-products occurs.

What is the nature of the interaction between the cell surface and glucose? It is obvious that no simple diffusion mechanism either through pores or through a lipid layer is compatible with the described properties such as the specificity, the kinetics, the temperature coefficient. The transport of sugars in the form of a complex with its liberation from the complex on the inside of the membrane might account for the specificity, kinetics and emperature coefficient, but could not readily account for aerobic-anaerobic differences, for the predetermination at the surface of the end products of metabolism, nor for the K^+–H^+ ion effects, which can be reproduced in a cell-free system in which the permeability barrier is destroyed.

The hypothesis described in the introduction to this chapter to the effect that sugar uptake involves metabolic interactions at the outer surface of the cell is consistent with the available data. It is suggested that the phosphorylation reactions of the fermentative schema are located in a structural element of the cell which lies directly underneath the permeability barrier (plasma membrane). This structural element may constitute the peripheral gelatinous zone of the cell generally called the cortex or ectoplasm. The integrity of the cortex remains intact in cell-free preparations even though the permeability barrier is destroyed as indicated by the failure of such a preparation to utilize sugar phosphates, even though it can rapidly ferment glucose. The fermentation by cell-free preparations also has a restricted pH range and a complete dependence on the presence of K^+; whereas that by live cells has a very wide range and no absolute dependence on potassium. Thus in a living cell the cortex must be shielded from the external environment. It is suggested that glucose is phosphorylated at the permeability barrier by ATP and hexokinase, that the sugar phosphate resulting from the reaction proceeds through the glycolytic reactions within the structure of the cortex, the ATP being regenerated by the coupled oxidation at the phosphoglyceraldehyde dehydrogenase reaction. This reaction also serves to pick up orthophosphate and could account for its uptake from the medium. It is assumed that sugar phosphates cannot penetrate beyond the permeability barrier, that orthophosphate can penetrate into the cortex and that glucose can move into the cortex only by undergoing phosphorylation. One reaction in glucose fermentation which is directly exposed to extracellular pH is the first one in the chain, the hexokinase reaction. It is suggested that in order to encompass the pH range 1·7 to 11·0, there must be two hexokinases with different pH optima explaining the biphasic curve for the effect of pH on fermentation, the different actions of Ca^{++} at high and low pH, and the relative rates of mannose metabolism. The effect of uranium is on the first reaction of sugar uptake by chelating with ATP and thus preventing the phosphorylation. The uranium does not penetrate into the cortex as shown by its failure to inhibit other metabolic reactions and by the fact that the uranium complex with the cell is directly susceptible to the effects of external pH. However, uranium also inhibits a second reaction, one which participates in respiration but not in fermentation. The effects of K^+ and NH_4^+ on fermentation seem to be due to their ability to counteract the inhibiting effects of external H^+ on fermentative enzymes. A similar effect can be shown with a cell-free fermenting system in which the permeability barrier is destroyed, and also on purified yeast hexokinase in the case of K^+ (unpublished data). Another fermentative reaction susceptible to K^+ and NH_4^+ is phosphohexokinase (Muntz, 1947).

This hypothesis is put forward on a tentative basis, with the realization that the existing data, although consistent with the general nature of the suggested mechanism, do not allow more than educated guesses concerning the details.

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ACTIVE CATION TRANSPORT IN ERYTHROCYTES

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I. INTRODUCTION

While the muscle cell has been regarded as typical of a living dynamic unit, the mammalian erythrocyte was for a long time considered to be a senescent structure specialized for oxygen carriage and subject to simple physical laws uncomplicated by vital processes. It is easy to understand how this view arose. The human erythrocyte, though rich in potassium and poor in sodium, survives for many days in a circulating plasma rich in Na and poor in K. Clearly, such a situation could only persist if the cell were impermeable to cations or if the paradoxical distribution were maintained by an active transport against the concentration gradients. It seemed improbable, however, that such a vital process could be attributed to a non-nucleated cell whose respiration was minimal, and so it was generally assumed that the anomalous distribution of cations arose as an active process in the immature nucleated respiring cell and was perpetuated in maturity by the cell membrane becoming impermeable to cations. In support of this view was the well-known observation that in short-term experiments mammalian erythrocytes in solutions of varying tonicities behave as osmometers. In short, then, the mature erythrocyte was regarded as a dead cell, permeable to simple anions, but impermeable to cations and to the cell and plasma proteins. On this basis the experiments of Henderson, Van Slyke and Gamble were founded, and though the fundamental assumptions were wrong, their observations conducted under controlled conditions are still valid and fruitful.

Between 1930 and 1940 several observers described the passive penetration of erythrocytes by Na (Jeanneney, Servantie & Ringenbach, 1939; Maizels & Whittaker, 1940) and also the passive escape of K (Dulière, 1931; Drew, Esdall & Scudder, 1939; Downman, Oliver & Young, 1940) and the observations, if acceptable, would imply as a corollary the existence of an active process to compensate for the passive cation movements. As, however, the experiments were carried out *in vitro* under highly artificial conditions, they were not held to invalidate the view that erythrocytes *in vivo*, or when freshly shed, were impermeable to cations. But in 1936, Henriques

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and Ørskov showed that when lead was injected into rabbits, the erythrocytes lost K and then gradually recovered the lost K as the effects of the injection passed off. The experiment is perhaps not quite conclusive since it is uncertain how much of the rise of cell K is due to actual uptake during recovery and how much is contributed by new-formed cells or by cells derived from reservoirs possibly inaccessible to lead. More conclusive were the experiments of Cohn & Cohn (1939) and of Maizels & Paterson (1940). The former showed that ^{24}Na injected into dogs rapidly exchanged with Na in the erythrocytes, while the latter authors, without using tracers, demonstrated permeability and active cation transport in the case of human erythrocytes in the following way. Group O blood was cold-stored until cell Na had risen to about 60 m.equiv./l., and 1000 ml. were then transfused to a group A recipient, causing an immediate rise of Na in the circulating cells from 14 to 32 m.equiv.; 6 hr. later, however, cell Na had fallen to 16 m.equiv./l., and since differential agglutination showed the donor cells to be still surviving, it followed that Na had left the donor cells against the concentration gradient. Moreover, since loss of Na was unaccompanied by cell shrinkage it was presumed that there had been a compensatory uptake of K—also against the gradient. The permeability of rabbit cells to ^{42}K was demonstrated *in vivo* by Hevesy & Hahn (1941) and to ^{24}Na by Mullins, Fenn, Noonan & Haege (1941) and also by Hahn & Hevesy (1942). Meanwhile, an impetus had been given to these studies by Steinbach (1940), who showed that excised frog's muscle lost K to K-free Ringer solution, regaining the K against a steep concentration gradient when only a small amount of K was added to the external medium. A year later (1941) Harris and also Danowski showed that, in stored blood, erythrocytes which had in the cold lost K in accordance with the concentration gradient regained K *in vitro* if incubated at 37° C. with glucose. Maizels (1949) confirmed these observations and also showed that Na gained during cold-storage was excreted during incubation.

Cation transport in erythrocytes has thus been established for just 12 years, and its metabolic basis and energetics subjected to much study. But in spite of the apparent simplicity of the mammalian erythrocyte, the nature and cause of cation movements across the cell wall remain quite obscure.

II. METABOLISM AND TRANSPORT

Both Harris and Danowski showed that glucose was essential to cation transport and that fluoride inhibited the active movements. Maizels (1951) showed that active transport was inhibited or abolished by fluoride and monoiodoacetate in high dilution, but not by poisons acting chiefly on the respiratory cycle, such as cyanide (10 mM), dinitrophenol (1 mM) and malonate (10 mM): Table 1 shows that fluoroacetate (10 mM) is also without effect; mepacrine and arsenite increase the permeability of erythrocytes but are probably without direct effect on transport. It follows from what has been said, that cation transport in human erythrocytes is based on energy derived from glycolysis and not from respiration: this is to be expected from the studies of Harrop & Barron (1928) and of Dische (1937) which identify red-cell metabolism with glycolysis. It is probable that transport in other mammalian erythrocytes is also based on glycolysis; this is so with rabbits and according to McKee, Ormsbee, Anfinsen, Geiman & Ball (1946) is also the case with monkey cells.

Energizing substrates. Glucose and mannose energize cation transport in high or low concentrations, while fructose is effective in high concentration (500 mg./100 ml.) but not in low (100 mg./100 ml.) (Maizels, 1951). The observations are surprising but accord with those of Meyerhof & Geliazkowa (1947) on the glycolysis of sugars by brain and sarcoma slices: here glucose and mannose are rapidly glycolysed in high or low concentrations, galactose is little affected, while fructose is rapidly glycolysed in 2% solution and only slowly in 0·2% solution. Meyerhof & Geliazkowa (1947) attribute this to differing affinities of the sugars for hexokinase. Galactose has little or no ability to energize active cation transport: neither has lactate nor pyruvate.

The findings in the case of pyruvate may seem to contradict those of Wilbrandt (1940); but this is not so and the subject requires further consideration. Wilbrandt, using massive amounts of fluoride (20–40 mM/l.), found that human erythrocytes shrink and become resistant to haemolysis by hypotonic solutions. The effect, fully developed in an hour or two, was presumably due to a rapid loss of K from the cells without corresponding gain of Na. It was inhibited by pyruvate—possibly through energy derived from the conversion of pyruvate to lactate. In Maizels's (1951) experiments much lower concentrations of fluoride were used (1·5–10 mM) in the presence of glucose: here some cell swelling was usually observed, though the writer remarks that this increase 'was sometimes less than would have been expected from the failure of active transport'. In any case it is clear that transport and glycolysis in human cells are inhibited by an amount of fluoride which is far less than that required to elicit the Wilbrandt effect, and this inhibition is not significantly affected by pyruvate. This finding is illustrated in Table 1 which also shows that, although inhibition of transport by fluoride is little if at all affected by pyruvate, the haemolysis which is constantly present in systems incubated with large or small amounts of fluoride is inhibited by the addition of pyruvate and this is observed consistently; lactate has no such effect.

The findings suggest the following conclusions: (1) Small amounts of fluoride (5 mM) produce maximal inhibition of glycolysis and transport while having little effect on the permeability of the cell membrane and such energy as may be liberated by the reduction of pyruvate cannot be used for cation transport (which may well depend solely on energy-rich phosphate bonds). The inhibition of transport results in a slow increase of cell Na and a gradual loss of K extending over many hours. (2) High concentrations of fluoride do not further increase inhibition of glycolysis or transport, but have an additional direct effect on membrane permeability, so that most of cell K is lost in an hour or two and is not immediately replaced by entering Na, whose rate of penetration is much less affected by the presence of fluoride. (3) The haemolysis found in the presence of low concentrations of fluoride and the marked increase in permeability to potassium which accompanies high fluoride concentrations are both mitigated by the presence of pyruvate, acting either in some simple physical way or by its reduction to lactate.

Table 1. *Cation transport in human erythrocytes: effects of fluoroacetate and of fluoride and pyruvate*

(Blood stored at 4° C. for 7 days and then incubated for 18 hr. External concentrations, K 10, Na 150 m.equiv./l. glucose 6 mM.)

No.	Hr. at 37° C.	Additions mM/l. cell suspension	Cells						
			Lysis (%)	V	pH at 20° C.	Contents (m.equiv./l. cells)*		Concentrations (m.equiv./l. cell water)	
						K	Na	K	Na
1*a*	0	(Unincubated)	0	105	7·24	66	54	88	72
b	18	None	0	99	7·20	91	24	132	35
c	18	None	0	101	7·11	89	24	126	34
d	18	Fluoroacetate 10	0	101	7·14	88	26	124	36
2*a*	0	(Unincubated)	0	103	7·02	52	61	71	84
b	18	None	0	100	6·93	74	34	106	49
c	18	NaF 4	1·2	106	7·12	46	70	61	93
d	18	NaF 4, pyruvate 28	0·4	104	7·20	47	68	63	92
e	18	NaF 4, lactate 28	1·0	104	7·22	45	70	61	94

V = cell volume as a percentage of the original cell volume.

* Contents corrected for changes in volume by reference to the original cell volume.

Nucleated erythrocytes

This account is based on unpublished work by the author.

Cation distribution in the erythrocytes of the chicken is very similar to that in man, but active transport in these nucleated cells may be energized either by lactate, pyruvate or glucose and when the two former substrates are added, fluoride and iodoacetate cause only a small decrease in active transport, any inhibition probably being non-specific and exerted on the respiratory cycle: for by contrast respiratory poisons like cyanide, carbon monoxide and dinitrophenol, and even simple deprivation of oxygen inhibit transport strongly, notwithstanding that glycolysis is active in the presence of these poisons.

Transport is not greater in the presence of glucose than it is with lactate, nor is the poisoning effect of cyanide enhanced by the addition of moderate amounts of fluoride or iodoacetate: both these observations show that active transport in chicken cells derives little or no immediate energy from glycolysis.

Cation transport in chicken erythrocytes thus has a respiratory basis, and in this resembles transport in brain and retina, where Terner, Eggleston & Krebs (1950) have found the process to be energized by glucose, lactate or pyruvate, but to require also the presence of glutamate. Thus, if potassium-depleted retina be incubated in a medium containing glutamate (5 mM/l.) and glucose, active transport of glutamate occurs, the tissue level rising from the normal of 6 mM to 20 mM/kg.; at the same time there is a movement of an equimolar amount of K into the tissues: in the absence of glutamate little or no cation transport occurs. In the chicken and man, however, erythrocytes contain less than 0·5 mM/l. glutamate, which if concerned at all in transport must act in some obscure cyclical fashion and certainly transport is active even in the complete absence of added glutamate.

Cation transport in the red cells of the grass-snake is qualitatively similar to that in the chicken, being based on respiration and not directly on glycolysis. The case of the tortoise is complicated. At 25° C. some degree of cation transport may be manifest, but at 37° C. there is a marked rise of cell Na and a moderate fall of K (both movements with the concentration gradients) and so the cells swell and may even haemolyse (Table 2). The effects are due to the use of a standard calcium-free medium for suspending the cells, and the addition of 3–5 mM Ca (probably less would suffice) greatly decreases loss of K and gain of Na. It is probable that Ca acts by decreasing cation permeability rather than by enhancing active transport, for were the action mainly on transport, the effects of calcium lack would be more marked at 23 than at 37° C. So, too, if cyanide is added to a suspension of tortoise erythrocytes at 25° C. cations move passively with the concentration gradient, but if calcium be present as well the passive penetration is prevented and cell K and Na remain practically unaltered (Table 2, nos. 1*a*, *d* and *e*): here, too, it must be presumed that in the cyanide-poisoned systems containing Ca, loss of respiratory activity and cation transport is offset by decreased permeability to cations. The effects of calcium on tortoise erythrocytes corresponds with its action on the erythrocytes of the snapping turtle (Lyman, 1945): here the cells swell and haemolyse in an artificial medium containing less than 1·7 mM Ca. Lyman found the phenomenon to be peculiar to the snapping turtle and absent in the golden-striped, box and marine turtles and also in the diamond-backed terrapin. In the case of carp's blood, Black & Irving (1938) have shown that the addition of oxalate promotes haemolysis, while fluoride has a similar effect on dogfish blood (Ferguson, Horvath & Pappenheimer, 1938), and also on the tautog (*Tautoga onitis*), sea robin (*Prionotus carolinus*) and squeteague (*Cyonoscion regale*) (Hamdi & Ferguson, 1940): Ferguson *et al.* suggested that removal of ionized magnesium was the cause of haemolysis in the bloods of these various fishes, but it is probable that Ca is the effective ion; certainly in the case of the tortoise Mg cannot replace Ca as an inhibitor of haemolysis. In my own experiments, cation transport in the erythrocytes of the frog, chicken and man were not affected significantly by the presence or absence of Ca: in the case of the tortoise, however, Ca is, as has been seen, an essential factor; it cannot be replaced by Mg, Cd, Ni or most other metals, while by contrast, Ba, Sr and (oddly) Co are as effective as Ca in preventing cell swelling and haemolysis.

Table 2. *Cation transport in tortoise erythrocytes: effects of temperature, calcium and cyanide*

(Blood stored at 4° C. for 7 days and then incubated. External concentrations, K 10, Na 160 m.equiv./l. glucose 11 mM.)

No.	Incubation		Additions mM/l. cell suspension	Erythrocytes						
				pH at 20° C.	V	Haemolysis	Contents (m.equiv./l. cells)*		Concentrations (m.equiv./l. cell water)	
	Temp. (° C.)	Time (hr.)					K	Na	K	Na
1 *a*		0	(Unincubated)	6·90	105	0	102	19	136	25
b	23	3	No addition	6·78	103	0	104	16	142	22
c	23	3	CaCl$_2$ 5	6·76	101	0	106	11	150	15
d	23	3	NaCN 2	6·65	112	0	94	30	115	37
e	23	3	NaCN 2, CaCl$_2$ 5	6·66	103	0	98	18	134	25
f	37	3	No addition	6·67	178	Marked	53	176	36	120
g	37	3	CaCl$_2$ 5	6·72	100	0	105	11	151	16

V = cell volume as a percentage of the original cell volume.

* Contents corrected for changes in volume by reference to the original cell volume.

So far then, we have encountered four types of cation transport in animal cells: one type in mammalian erythrocytes based on anaerobic glycolysis, a second type in chicken and snake erythrocytes based on respiratory activity and unaffected by Ca, a third type seen in the African tortoise (no other variety was investigated), the snapping turtle and a variety of teleosts and elasmobranchs is also probably based on respiration, but requires the presence of calcium to control permeability; the fourth type of transport, also aerobic, is seen in brain, retina and other tissues, and requires the presence not only of oxidizable substrate, but also of glutamate. With regard to the third type of transport, it is possible that a survey of erythrocyte susceptibility to Ca-lack might reveal interesting relationships between different species.

Sites of cation transport in erythrocytes

In non-nucleated erythrocytes the mechanism for cation transport is presumably located in the cell membrane because this is the sole site for those phosphorylations on which transport depends. Since no phosphorylation occurs at the outer cell face, but only dephosphorylation (Clarkson & Maizels, 1952) it may be assumed that K transport from without inwards must be preceded by a physical penetration of the cell surface before the cation carriers concerned can become effective. In the case of chicken erythrocytes, it has been seen that transport is based on respiration. But the cells do not stain with janus green and appear to contain no mitochondria, though it is possible that the respiratory apparatus is diffusely disposed in the cell membrane; in any case the actual transport must obviously take place across the cell membrane, and it is here that cation carriage and transport must be activated even in those cells where the energizing enzyme systems can be localized to respiring mitochondria within the cell.

Energy for transport

Raker, Taylor, Weller & Hastings (1950) found that 1·5 mM glucose were metabolized by 1 l. cells in 1 hr. when the pH of the suspending medium was 7·5; at pH 7·0 the figure was 0·93. Maizels (1951) gives 1·5 mM at cell pH 7·4 (7·6 external), calculated to be more than ten times greater than is needed to account for the observed cation transport. It is probable that the transport depends on the presence of energy-rich phosphate bonds and there is no evidence in favour of Solomon's (1952) suggestion that the energy derives from the reduction of pyruvate to lactate in the presence of coenzyme 1. This view is based on a misconception of the Wilbrandt effect which, as has been shown, is primarily concerned with the effects of fluoride and pyruvate on cation permeability and not on transport.

III. THE MOVEMENTS OF CATIONS IN HUMAN BLOOD

Dean (1941) first stressed the idea of a cation pump for muscle. He wrote: 'If potassium and sodium are mixed inside the fiber as free ions, then the pump that builds up the internal concentration of potassium must be pumping potassium in or sodium out or both.' He discusses especially the Na pump, but continues later: 'it makes little difference whether potassium or sodium is pumped.' In the case of muscle, Krogh (1946) suggests the existence of a Na pump, but of the erythrocyte he says: 'No evidence is available to show whether Na or K or only one of these ions is actively transported, but a K transport appears most likely.' Elsewhere, however, it was remarked (Maizels, 1949) that the physical results of a large amount of non-penetrating anion (haemoglobin and organic phosphate) within the erythrocytes were such as to tend to swelling and rupture—a tendency which would be accentuated by the presence of an inwardly acting K pump and which could not be adequately opposed by any purely physical process acting on the sodium ion. He further pointed out that an outwardly acting sodium pump, on the other hand, would suffice to overcome the Donnan effect of non-penetrating cell anion and might even determine such a backflow of K that at equilibrium the cation distribution characteristic of the human erythrocyte was attained. Solomon (1952) criticizes this view on the grounds that at equilibrium Na leaving the cell equals Na entering and so no free energy is left for K transfer. The observations, however, are irrelevant, for the cold-stored blood systems considered by Maizels were not at equilibrium during the early stages of incubation and with cell Na actually falling at the rate of 2–3 m.equiv./l. per hour against a steep concentration gradient, a backflow of K is possible. However, in systems at equilibrium Solomon ignores completely the Donnan effect of non-penetrating cell anion which, with Na 'fixed' by transport at a constant low level, must tend to attract K and water instead of Na and water.

But while Solomon's criticism is not valid, the simple theory of Na transport with compensatory movement of K is unacceptable on other grounds (Davson, 1951; Harris & Maizels, 1952). Thus, if the distribution of K were secondary to that of Na and governed by potentials arising from the presence of non-penetrating cell anion, $[\mathrm{K}]_{\mathrm{cell}}/[\mathrm{K}]_{\mathrm{plasma}}$ should equal $[\mathrm{H}]_{\mathrm{cell}}/[\mathrm{H}]_{\mathrm{plasma}}$ and $[\mathrm{Cl}]_{\mathrm{plasma}}/[\mathrm{Cl}]_{\mathrm{cell}}$. Harris & Maizels (1952) have in fact found good agreement between the hydrogen and chloride ion ratios, the actual values depending on the pH and not on the metabolic state of the cells. But the potassium ratio is very different, for in fresh human blood $[\mathrm{Cl}]_{\mathrm{plasma}}/[\mathrm{Cl}]_{\mathrm{cells}} = 1{\cdot}4$, while $[\mathrm{K}]_{\mathrm{cell}}/[\mathrm{K}]_{\mathrm{plasma}} = 30$. The con-

Table 3. *Cation transport in human erythrocytes*

(Exp. A: blood stored 7 days at 4° C. and then incubated in solutions containing glucose 8 mM, KCl 10 m.equiv./l. and varying proportions of NaCl and LiCl. Exp. B: fresh blood incubated in NaCl-LiCl mixtures.)

Exp.	Hr. at 37° C.	Cells						External medium			
		V	pH at 20° C.	Contents (m.equiv./l.)*		Concentrations (m.equiv./l. water)		Concentrations (m.equiv./l. water)			$[Na]_i/[Na]_e$
				K	Na	K	Na	K	Na	Li	
A1	0	101·5	7·11	69·5	48·5	97·5	68	10	142	0	0·48
	4	98	7·10	71·5	39	106	58	10	142	0	0·41
	11½	98	7·26	75·5	32	112	47	10	142	0	0·34
	24	97	7·02	82	24	122	36	10	142	0	0·25
	27	100	7·15	81·5	24	117	34·5	10	142	0	0·24
A2	0	101·5	7·11	69·5	48·5	97·5	68	10	53	89	1·28
	4	96	7·11	68	30·5	103·5	46·5	10	53	89	0·88
	11½	97	7·28	72	19·5	108	29	10	53	89	0·55
	24	98	7·06	72	11·2	106	16·5	10	53	89	0·31
	27	98	7·09	71	10·5	104·5	15·5	10	53	89	0·29
	30	99	7·10	70	10·3	102	15	10	53	89	0·28
B1	0	100	7·40	101	12·0	144	17·2	10	145	0	0·12
	6	96	7·27	99·5	11·6	151	17·5	10	145	0	0·12
	24	97	7·21	102	11·2	152	16·7	10	145	0	0·12
	30	100	7·20	105	10·6	151	15·2	10	145	0	0·11
	47	101·5	7·28	103·5	12·6	145	17·9	10	145	0	0·12
B2	0	100	7·40	101	12·0	144	17·2	10	70	75	0·24
	6	96	7·27	96·5	7·7	146	11·6	10	70	75	0·17
	24	101	7·19	90·5	7·5	128	10·6	10	70	75	0·15
	30	101	7·18	89·5	5·6	126	7·9	10	70	75	0·11
	47	103·5	7·26	88·5	6·6	120	9·0	10	70	75	0·13

Exp. A1, $k_{out}=0·11$ hr.$^{-1}$; $k_{in}=0·027$ hr.$^{-1}$. Exp. A2, $k_{out}=0·13$ hr.$^{-1}$, $k_{in}=0·028$ hr.$^{-1}$.
V = cell volume as a percentage of the original cell volume.

* Contents corrected for changes in volume by reference to the original cell volume. Note: cell contents and concentrations corrected for the Na content of intercellular fluid.

Table 4. *Cation movements in the cells of human blood stored at 4° C. for 1 week with LiCl 135 and Na 15 m.equiv./l. water and then incubated in mixtures of KCl and LiCl containing glucose 6mM for 18 hr.*

(Control blood (Exp. 2) stored at 4° C. in NaCl 150 m.equiv./l. water and incubated with KCl and NaCl.)

No.	Cold stored in	Hr. at 37° C.	Cells							Incubation medium		
			pH at 20° C.	Contents (m.equiv./l. cells)*			Concentrations (m.equiv./l. water)			Concentrations (m.equiv./l. water)		
				K	Na	Li	K	Na	Li	K	Na	Li
1*a*	LiCl	0	6·96	73	8	33	100	11	46	—	—	—
b	LiCl	18	7·16	64	2·6	52	94	4	76	0	0	160
c	LiCl	18	7·16	63	2·4	52	93	3·5	76	10	0	150
d	LiCl	18	7·07	69	2·2	45	102	3	66	22	0	138
e	LiCl	18	7·03	81	2·4	37	112	3·5	52	83	0	77
f	LiCl	18	7·06	90	2·5	28	129	3·5	40	117	0	43
g	LiCl	18	7·01	92	2·3	24	132	3·5	35	160	0	0
2*a*	NaCl	10	7·02	52	61	0	71	84	0	—	—	—
b	NaCl	18	6·93	74	34	0	105	49	0	10	150	0

* Contents corrected for changes in volume by reference to the original cell volume.

clusion is thus enforced that cation distribution in blood involves a transporting mechanism for K in addition to that for Na. There is no evidence, however, that the two mechanisms are independent, and it is more likely that they are closely integrated: thus, in sodium-depleted erythrocytes where further net output of Na is limited, little uptake of K is manifest (Table 4); so, too, when cells are incubated in media of exceptionally low K content, significant uptake of K is no longer possible, and at the same time output of Na ceases to be apparent. These matters are discussed later: in the meantime it is interesting to note that though a simple Na transporting device acting alone might suffice to give blood its characteristic distribution of Na and K, the cells in the absence of a simultaneous active transport of K would be so acid and so lacking in chloride and bicarbonate ions as to function imperfectly as units for buffering and oxygen carriage (Harris & Maizels, 1952).

Sodium

In what follows, the word 'transport' will be used for movements against gradients of concentration and potential, 'transfer' will be used for movements in any direction and 'diffusion' for purely passive movements.

Transfer constants may be obtained most accurately by means of tracers, or in the case of Na the 'chemical method' of Harris & Maizels (1951, 1952), which requires no radio-sodium, may be used. In the latter type of experiment, blood is first cold-stored and then incubated with glucose so that the decline in cell Na concentration may be estimated: the volume of the external phase is always very large, so that for practical purposes its cation concentration may be regarded as constant throughout the experiment. In either case (but especially in the 'chemical method') error is introduced by decreased permeability which occurs on incubating erythrocytes (Sheppard, Martin & Beyl, 1951; Harris & Prankerd, 1953), and by failure of metabolism resulting from dephosphorylation. As a result, the curve for log ^{24}[Na] against time is only linear for a few hours, tending to 'flatten' thereafter and to give the impression that only part of cell Na is freely exchangeable. According to data obtained *in vitro* at 37° C. by Solomon (1952) about 3 m.equiv. cell Na are unexchangeable or exchanged very slowly. Solomon contrasts this with his own findings at lower temperatures and with the results *in vivo* of tracer experiments communicated to him personally by Edelman, James & Moore, where in either case cell Na appeared to be fully exchangeable. Solomon concludes: 'the present results showing a more slowly exchangeable Na fraction *in vitro* must be accepted with reserve and are certainly not indicative of the true state in nature.' Using both the chemical and tracer methods for human erythrocytes in phosphate-NaCl media at 37° C., Harris & Maizels (1951) obtained k_{out} (the outward transfer constant) as 0·25 and k_{in} (the inward transfer constant) as 0·023 hr.$^{-1}$. Solomon (1952), using a $NaHCO_3$-NaCl medium, obtained k_{out} 0·6 hr.$^{-1}$ and k_{in} 0·022 hr.$^{-1}$. Solomon's higher figures were due in part to his allowance for the slowly exchangeable Na fraction in the cells, but also to the superiority of his medium, for with a medium similar to Solomon's figures of 0·3 hr.$^{-1}$ have been obtained for k_{out}. Even so, results for the transfer constants of Na obtained with the phosphate-NaCl medium have a relative, if not an absolute, value. Certain other findings will now be reviewed.

The ratio of cell and external sodium concentrations. The investigation is complicated by the necessity for adding substances like lithium chloride or sucrose when lowering the external sodium concentration, if hypotonicity is to be avoided. Flynn & Maizels (1949) cold-stored blood for several days so that cell Na was high, and then measured the fall of cell Na on incubating the cells in relatively large volumes of solutions containing glucose and KCl (10 m.equiv./l.), together with NaCl and LiCl in varying proportions: they found that after about 24 hr. the ratio of cell to external sodium concentrations tended to be constant. Harris & Maizels (1951) prepared time curves for Na output on incubation and confirmed by extrapolation to $t = \infty$, that $[\text{Na}]_{\text{in}}/[\text{Na}]_{\text{out}}$ was approximately constant as was $k_{\text{out}}/k_{\text{in}}$. The individual values of the constants may vary, though in fact in actively transporting bloods k_{out} and k_{in} are both little affected by variations in external Na. The findings conflict with those of Solomon (1952) who reports that lithium has no effect on K transfer, but may decrease Na transfer by 30% or more. It may be noted, however, that the absolute changes effected by Li in Solomon's transfer figures are in fact quite small: k_{out} alters from 0·319 to 0·274 hr.$^{-1}$ and k_{in} from 0·00874 to 0·0102 hr.$^{-1}$. The original experiments on the Na output of stored cells have therefore been repeated (Table 3): it will be seen that the transfer constants of Na are little affected by the presence of Li, whose entry into the cell nevertheless decreases by about 10 m.equiv./l., the final level attained by K in the cells. So, too, when external Na is decreased and tonicity is maintained by use of K, $[\text{Na}]_i/[\text{Na}]_e$ still tends to constancy (Table 5).

External potassium concentration and sodium transfer. Variations of external K between 14 and 4 m.equiv./l. (Solomon, 1952) and even down to 2 m.equiv. (Harris & Maizels, 1951) have little effect on the transfer constants for Na, but with $[\text{K}]_e$ below 1 m.equiv. both constants and especially k_{out} fall, the Na efflux being depressed, and it may well be, as Flynn & Maizels (1949) suggest, that in the theoretical (but hitherto unattained) K-free system, Na efflux would cease altogether.

pH *and cell sodium*. Between pH 7·2 and 7·6 (cells) output of Na from stored cells is maintained at a steady level (Flynn & Maizels, 1949). Below pH 7·1 the manifest output falls, presumably because of decreased efflux, while above pH 7·7 manifest output is also decreased, probably as a result of increased influx: the value of the latter observations is uncertain because of the haemolysis which becomes apparent as cell pH exceeds 7·7.

Lithium

Data from Table 4 (where K and Na were estimated chemically and Li with a flame photometer) suggests that in sodium-poor cell suspensions at 37° C. k_{in} and k_{out} for Li are, over a period of 18 hr., about 0·016 hr.$^{-1}$. Similarly, when cells depleted of Na by cold-storage in LiCl solutions were incubated in glucose-containing media rich in Na and Li, but poor in K, the inward transfer constant determined from direct measurement of Li was found to be about 0·022 hr.$^{-1}$ (Table 5). Without attributing any great degree of accuracy to these figures, they do suggest that the passive transfer rate for Li is of the same order as those of Na or K. The data also show that there is no active efflux of Li comparable to that occurring with Na. As in the case of Na, however, the rate of passive influx is not constant throughout the incubation period, but decreases with time. Thus in Table 3, the concentrations of Na+K in Exp. B1 (where total base concentration is fairly constant throughout) compared with the concentrations of Na+K in Exp. B2, suggest that in the latter the concentrations of Li at 0, 24 and 47 hr. are about 0, 23 and 32 m.equiv./l. cell water respectively. Hence, the transfer constant for influx in the first 24 hr. is about 0·017 hr.$^{-1}$, while between 24 and 47 hr. it is about 0·007 hr.$^{-1}$. Other experiments of this type show similar reductions of Li influx with time.

Table 5

Cell Li (m.equiv./l.)		External concentration			Transfer constant for Li influx at 37° C.
At 0 hr.	At 18 hr.	K	Na	Li	
27	26	10	120	25	—
28	36	10	95	50	0·024
29	43	10	70	75	0·020

Potassium

According to Dean, Noonan, Haege & Fenn (1940) about 1·4% of cell K exchanges per hour; Raker *et al.* (1950) give 1·6% at 37° C. and Sheppard & Martin (1950) 1·8% at 38° C.

External sodium and cell potassium. When external Na is lowered, tonicity being maintained by LiCl, uptake of K by cells incubated after cold-storage is decreased: this is shown in Table 3. In five similar experiments reduction of external Na from 140 to 50 m.equiv. (with increase of external Li from 0 to 80 m.equiv.) decreased the average gain of K by 62%, while it increased the loss of Na by 43%; similar figures are reported by Ponder (1950).

If it be assumed that cell K in Table 3 is approaching equilibrium in 24–30 hr., then the data for stored cells suggests that when external Na is lowered from 142 to 53 m.equiv./l. K influx falls by about 15%; in the case of the fresh cells in Exp. B, decrease of $[\mathrm{Na}]_e$ from 145 to 70 m.equiv. lowers the equilibrium value of $[\mathrm{K}]_i$ (at 30 hr.) from 151 to 126 m.equiv./l. cell water; assuming that the rate constant for K efflux remains unaltered or changes similarly in the KCl-NaCl and KCl-NaCl-LiCl systems during incubation, then halving $[\mathrm{Na}]_e$ reduces K influx by about 16%.

Cell sodium and cell potassium. Flynn & Maizels (1949) remarked that if conditions during cold-storage (e.g. in a LiCl medium) were such as to cause cell Na to fall to a low level, little further active output could occur during the subsequent incubation, and under these circumstances there is practically no active uptake of K. Thus in Table 4 of the present paper, where Na output during incubation is only about 5 m.equiv./l. cells, uptake of K does not exceed this figure so long as $[\mathrm{K}]_e$ lies between 0 and 22 m.equiv. and it is not until $[\mathrm{K}]_e$ approaches 80 m.equiv. that definite increase in $[\mathrm{K}]_i$ occurs. It is thus clear that when Na efflux falls, K influx is also decreased. The findings contrast with those of the control experiment (Table 4, no. 2), where cells from the same blood cold-stored and incubated in Na-rich media show very active movements both of Na and K. It should be noted that decrease in K transport can only be demonstrated when both erythrocytes and suspending medium are depleted of Na before incubation: if the medium be Na-poor and the cells Na-rich, output of Na and uptake of K will still occur, and this has led Ponder (1950) to state that K transport remains active in 'LiCl and CsCl systems'. In short, Ponder's experimental conditions correspond to those already described in the preceding section.

It has previously been noted that Na transport depends on an adequate amount of K in the external medium and the present argument shows the correlation of K transport with Na efflux: thus the integration of the various cation movements is close.

Concentration of cell potassium and the magnitude of the potassium influx. Under normal conditions K influx amounts to about 1·6 m.equiv./l. cells $\mathrm{hr.}^{-1}$ at 37° C. (Raker *et al.* 1950; Sheppard & Martin, 1950; Solomon, 1952), but when stored blood with K-depleted cells is incubated with glucose the influx in active preparations may exceed 2 or even 3 m.equiv. $\mathrm{hr.}^{-1}$ (for examples see Flynn & Maizels, 1949; Ponder, 1950). Such a high rate is only maintained for a few hours, the influx tending to fall as cell K rises and perhaps also as a result of metabolic failure. Hence, in our experiments over a period of 18 hr. incubation, increase of cell K was about 1·2 m.equiv. hr.$^{-1}$, averaging 1·4 for sixteen stored bloods with a mean cell K content of 62 m.equiv./l. at the end of cold-storage and 1·0 for thirteen bloods whose mean cell K was 75 m.equiv.; it thus appears that the lower cell K is at the beginning, the greater the rise during incubation.

External potassium and cell potassium. In the metabolizing erythrocyte increase in the external K concentration, $[K]_e$, might affect cell K by increasing adsorption, or else by displacing cell Na, or by increasing the passive entry of K into the cell, or by affecting the active K influx. The effect of K adsorbed is very slight. Cells suspended in simple KCl solution (0·175 M) gain about 6 m.equiv./l. K almost immediately, subsequent gain being much slower (Maizels, 1935): assuming that this quick gain is due to adsorption and allowing for K in the intercellular fluid, this gives a probable figure for K adsorbed of about 2 m.equiv. Hence, with external K at 75 m.equiv./l. this immediate rise, attributed to adsorption, should be very small: experimentally, the net value was found to lie between 0·5 and 1 m.equiv./l. cells.

Displacement of cell Na is more significant. Since, as we have seen, $[Na]_i/[Na]_e$ is constant, rise of $[K]_e$ from 5 to 75 m.equiv., with fall of $[Na]_e$ from 140 to 70 m.equiv./l. will about halve the concentration and also the content of cell Na (Tables 3 and 6); assuming that K influx is linked with and equal to about half of Na efflux, increase of cell K directly due to displacement of 4–8 m.equiv. Na, will be about 2–4 m.equiv./l. cells in 24 hr.

Passive influx is negligible under physiological conditions with plasma K set at the low level of 5 m.equiv./l.; so, too, passive efflux of Na may be ignored when cell Na is only about 12 or 15 m.equiv./l. But in artificial systems with $[K]_e$ or $[Na]_i$ raised to a high level, passive influx of K or efflux of Na become significant. This aspect is usually ignored, with the implication that the whole of K entry into and Na exit from the metabolizing cell is active.

It is true that there is no direct evidence of passive K penetration into normal erythrocytes, for though cells depleted of K during cold-storage and then incubated in glucose-free K-rich media permit the passive entry of K at a rate comparable to passive K efflux from the metabolizing cells (0·01–0·02 hr.$^{-1}$), this does not prove that there is a passive entry of K under physiological conditions. But since in the actively metabolizing cell Li enters and leaves at about the same rate, while passive penetration of Na is also free, it seems likely that under these conditions passive influx of

K also occurs; indeed, it is unlikely that a membrane passively permeated by an ion in one direction, would be impermeable in the reverse direction. Hence, it may be assumed that the rate constant for passive influx of K(R) equals that for passive K efflux (E), provided that allowance is made for the Donnan asymmetry. The passive influx may then be calculated from Harris's (1953) equation, $R=f^2\times E\times[\mathrm{K}]_e/[\mathrm{K}]_i$, where $[\mathrm{K}]_e$ and $[\mathrm{K}]_i$ are the respective external and internal concentrations and f is the asymmetry factor of Harris & Maizels (1952) which equals $[\mathrm{Cl}]_e/[\mathrm{Cl}]_i$ or about 1·14 at the usual experimental pH. Thus, with $[\mathrm{K}]_e$ at 5 m.equiv./l. it may be calculated that the active moiety of influx is 1·54 and the passive 0·06 per l. cells hr.$^{-1}$; giving the observed total influx of 1·6 m.equiv., at which level the K content of normal cells remains constant with a rate for outward transfer of 0·016 hr.$^{-1}$. But with $[\mathrm{K}]_e$ at 75 m.equiv. K influx should rise from 1·52+0·08 m.equiv. to 1·52+1·1 or 2·62 m.equiv. hr.$^{-1}$, and cell K should rise by about 22 % during the 24 hr. incubation. Observed increases are shown in Table 6, and after making the appropriate deductions for K adsorbed and K displacing Na, the total net influx in 24 hr. is about

Table 6. *Effect of external potassium concentration on the potassium content and concentration of human erythrocytes*

(Fresh blood incubated in mixtures of KCl and NaCl containing glucose.)

Exp.	Buffer	Hr. at 37° C.	Cells						External medium. Concentrations (m.equiv./l. water)	
			V	pH at 20° C.	Contents (m.equiv./l.)*		Concentrations (m.equiv./l. water)			
					K	Na	K	Na	K	Na
1	Phosphate	0	100	7·31	96	15	138	21	5	150
		0	100	7·35	97	14	140	20	75	80
		24	96	7·37	95	14	144	21	2	153
		24	97	7·27	97	11	145	16	5	150
		24	98	7·25	102·5	9·5	151	14	25	130
		24	101	7·23	112	6·1	157	8·5	75	80
2	$NaHCO_2$	0	100	7·36	98	11·9	140	17	5	150
		0	100	7·40	99	11·3	142	16·2	75	80
		24	97	7·49	98	12·8	146	19	5	150
		24	102	7·57	116	6·9	160	9·5	75	80
3	$NaHCO_2$	0	100	7·40	101	11·1	145	15·8	5	150
		0	100	7·44	101·5	9·8	146	14·0	76	80
		24	98	7·45	101·5	9·5	149	14·0	5	150
		24	104	7·45	117·5	5·7	159	7·7	75	80

V=cell volume as a percentage of the original cell volume.

* Contents corrected for changes in volume by reference to the original cell volume.

Note: cell contents and concentrations corrected for Na and K contents of the intercellular fluid.

13 m.equiv., corresponding to a reduction in the active component of influx of about 25 %, when $[K]_e$ rises to 75 m.equiv./l. and $[Na]_e$ falls from 145 to 75 m.equiv.: it has already been seen that if $[Na]_e$ is similarly reduced by the addition of LiCl (instead of KCl), active influx of K suffers a similar but smaller reduction of about 15 %.

These findings may now be compared with those of other workers. Flynn & Maizels (1949) found that when stored cells were incubated in media containing glucose the rise in cell K was rather greater with $[K]_e$ at 25 than at 5 m.equiv./l.; owing to increase in cell volume, however, the increase in cell K concentration was relatively less: this is also evident in Table 6 of the present paper. Davidsen & Kjerulf-Jensen (1950) also report increased K influx with rise of $[K]_e$. Solomon (1952), on the other hand, working with a range of $[K]_e$ between 4·5 and 16·75 m.equiv./l. states that (total) K influx is unaffected by external K concentration, though his results show a *reduction* in total influx in three of five experiments; in one case rise of $[K]_e$ from 9·35 to 11·35 m.equiv. lowered the transfer rate for K by 22 %: it should perhaps be noted that most investigators of the effects of $[K]_e$ on influx have limited the external concentration of K to between 5 and 20 m.equiv., which could in any case cause little rise in total influx—certainly not more than 15 %. Indeed, the only evidence that K influx is unaffected by *very* large rises in $[K]_e$, is limited to a single record in the paper by Raker *et al.* (1950). The table below is modified from their table 6 and the figures in columns 1 and 2 are from Raker's and his co-workers' own data:

Table 7

$[K]_e$ m.equiv./l.	mM K exchanging per l. cells $hr.^{-1}$ at 37° C. (total influx)	Passive influx	Active influx
4·47	1·67	0·07	1·60
38·5	1·33	0·62	0·71
74·4	1·58	1·19	0·39

The data evoke the following comments: comparison of the figures for total influx with those for $[K]_e$ suggests that the experimental error must be high, for increase of $[K]_e$ from 4·47 to 38·5 m.equiv. appears to decrease total K influx by 20 % and active K influx by 50 %, while increase of $[K]_e$ from 4·47 to 74·4 m.equiv./l. seems to depress total influx by only 6 %, while lowering the true active influx by no less than 75 %. In the absence of further supporting data and in view of the findings in this paper and in that of Davidsen and Kjerulf-Jensen it would seem reasonable to conclude that with marked rise in the external K concentration there is some small depression of true active K influx, and as a result total K influx is a little less than would be expected from the data for passive K permeation. This matter is considered again later.

pH *and potassium transfer.* According to Raker *et al.* (1950) change of external pH between 7 and 7·7 leaves K influx unaffected, but as the particular system beginning at pH 7·7 ended at pH 7·2, the significance of the range indicated is doubtful, and a range of 7–7·5 is more probable. Flynn & Maizels (1949) found net uptake of K during the incubation of stored cells to vary rather little for a range of cell pH between 7 and 7·6, though there was a definite maximum at pH 7·3 (pH readings at 20° C.); Ponder's (1950) figures are similar.

Temperature coefficients

According to Solomon (1952) the apparent energy of activation for K transfer is about 12,300 calories/mole, and he derives the following values from the respective data of Raker *et al.* (1950), Sheppard & Martin (1950) and Ponder (1950): 14,500, 15,800 and 16,200. In the case of Na transfer across the erythrocyte membrane, Solomon gives 20,000 for the activation energy of influx and 15,000 for efflux, from which it follows that cell Na concentration must be less at 25° C. than at 37° C. Actually, the reverse is the case, and in a recent experiment with stored blood $[Na]_i$ fell from 65 to 33 m.equiv./l. cell water after 24 hr. incubation at 37° C. and only to 50 m.equiv. at 25° C.; it follows that the activation energy for Na efflux must in fact be greater than for influx. According to Harris (1953), the activation energy for the passive fluxes of Na and K equal 14,000 cal./mole, while the value for the active fluxes of Na and K is nearly twice as great. Ponder (1950) and also Solomon (1952) remark that the energy of activation for K transport and for glycolysis is similar and they imply that the correspondence arises from the interrelation of transport and glycolysis. This may well be true for active cation transport, but the high energy of activation for passive movements does not necessarily suggest that cations are here also combined with chemical carriers, and the high energy of activation may well be a physical result of the difficulty with which cations penetrate the cell wall barrier (see Danielli & Davson, 1934). The matter is of importance because there is still a tendency to ascribe any reaction with a high activation energy to chemical processes. Thus, Solomon (1952) states that 'the high temperature coefficient observed by Gourley & Gemmill (1950) for phosphate transfer is typical of a metabolically linked process rather than simple diffusion'—a view which Gourley & Gemmill themselves advance. In the case of erythrocytes suspended in pure isotonic phosphate solutions the activation energy calculated from Maizels's (1932) data is 16,000—a figure which holds both at pH 8 and 5·4. This independence of pH suggests that neither phosphorylation nor dephosphorylation are major factors in the penetration of phosphate from pure phosphate media, and this view is supported by the speed of entry at pH 5·4 which with an external phosphate concentration of 130 mM amounts to 21 mM/l. cells per min. at 37° C. With erythrocytes in 6·9% glucose entry may exceed 30 mM glucose per min., a rate which would seem to exclude a chemical basis for glucose transfer; the energy of activation was 18,000; Masing's (1914) figure was 14,000. In the case of ox cells, the activation energy for penetration of polyhydric alcohols may rise as high as 23,000 (Jacobs, Glassman & Parpart, 1935). Hence, it is necessary to be cautious when ascribing a chemical basis to a phenomenon on account of its high energy of activation.

Transfer constants of various species

Most of these observations were made between 1939 and 1942, and in view of complicating factors such as slowly exchanging Na (Sheppard *et al.* 1951) and K (Hevesy & Hahn, 1941) their significance is uncertain. However, data collected from various sources by Sheppard *et al.* shows that in the cow and sheep, as in man, Na penetrates the erythrocyte membrane more readily than K. So too, in the case of dog erythrocytes, Krogh (1946), using the data of Cohn & Cohn (1939) and of Hahn & Hevesy (1942), showed that here also Na penetrates more readily than K.

In the case of chicken erythrocytes, the transfer constants of Na at 25° C. (measured by the chemical method of Harris & Maizels, 1951) are as follows: at pH 6·8, $k_{\text{out}} = 0{\cdot}45$; at pH 7·2–7·75, 0·7 and at pH 7·7, 0·6 hr.$^{-1}$: k_{out} for human cells in a similar medium (NaCl solution buffered with phosphate) was 0·06 at pH 7·3 and 25° C. k_{in} for chicken cells at 25° C. varied between 0·03 and 0·05 (four experiments). Taking an average figure for chicken erythrocyte volume and area of $130\mu^3$ and $180\mu^2$, the permeability constants are found to be for efflux $4{\cdot}9 \times 10^{-5}$ and for influx $2{\cdot}0 \times 10^{-6}$/cm. hr.$^{-1}$.

IV. GENERAL CONSIDERATION OF CATION TRANSPORT IN HUMAN ERYTHROCYTES

The cell wall barrier

It has been seen that in the metabolizing human erythrocyte the active and passive fluxes for Na exceed the corresponding K fluxes; the same holds for the erythrocyte of the dog and for other, if not all, mammalian erythrocytes. So, too, in the case of the non-metabolizing human cell (kept at 4° C. or incubated in the absence of glucose) Na penetrates more rapidly than K, and the naturally K-rich cells suspended in a Na-rich medium swell. This last observation is complementary to that of Davson & Reiner (1942) on the cat: cat erythrocytes are naturally poor in K and rich in Na and if suspended in KCl solutions show an excess of Na lost over K gained. So, too, when tortoise cells are suspended in a calcium-free NaCl solution, gain of Na is thrice as fast as the simultaneous loss of K.

Since of the two hydrated ions Na is the larger, it is likely that Na and K penetrate unhydrated, presumably through a non-watery lipoid phase and possibly combined with lipoid soluble carriers. This possibility has been advanced by Davson & Reiner (1942) and by Solomon (1952). It is interesting to recall that according to Teorell (1952) erythrocyte ghosts offer considerable resistance to the passage of cations, a resistance which is much decreased by the addition of oleate; nevertheless, even in the absence of oleate, K appears to penetrate more quickly than Na. Teorell considers that the cation penetration of ghosts is compatible with passage in a watery solution through a positively charged membrane, enhancement of passage by oleate being due to a decrease of positive charge. It has already been seen that in certain circumstances K may cross the membrane of the intact human erythrocyte more rapidly than Na; this occurs in the presence of lead or of high concentrations of fluoride, and possibly these poisons effect a phase reversal similar to that which may occur in the membrane of the erythrocyte after haemolysis.

Cation carriage

Before proceeding with the discussion of this subject, it will be well to recall the phenomena characteristic of transfer in the human erythrocyte. (1) The rate of passive transfer of Na is greater than that of K, while active transfer of Na also exceeds that of K. (2) During cold-storage, cell Na, total base, water and volume all increase, while during subsequent incubation with glucose all these changes are reversed and cell Na, total base, water and volume all decrease. The greater the increase in base at 4° C., the greater is the decrease at 37° C. (3) In cell suspensions, the ratio of the Na concentrations in cells and suspending medium is constant and independent of the external Na concentration (provided that $[K]_e$ exceeds a certain minimum critical value. (4) When external K is reduced below this critical value (1–2 m.equiv./l.) manifest output of Na and uptake of K fail. (5) As external K is raised above the critical value, further increase in Na efflux and K influx becomes relatively small and since with high $[K]_e$ some cell swelling occurs, the concentration of cell K is even less affected. (6) When, as a result of cold-storage in a Na-free medium, cell Na falls to a very low level, output of Na during subsequent incubation is necessarily limited and in these circumstances uptake of K is much reduced. (7) In cells cold-stored in a Na-rich medium, Na is high and K low; when such cells are incubated Na efflux and K influx both increase and may be twice as great as in cells whose composition is normal. Of these observations, Krogh (1946) especially has emphasized the first; the last was derived by Harris & Maizels (1952) from data presented by Flynn & Maizels (1949) and Ponder (1950). The remaining observations were made by Flynn & Maizels (1949) as a result of direct chemical analysis and those relating to Na were confirmed by Harris & Maizels (1951) using tracers.

It may be said at once that the theory recently applied to frog's muscle by Ling (1952) is not applicable to cation transport in erythrocytes. Ling supposes that in the presence of certain energy-rich phosphate compounds, the ability of myosin to adsorb ions is much increased, K then being preferentially adsorbed for purely physical reasons. There is thus a transfer of K from one phase, the extracellular medium, to a second phase, the muscle cell which is considered to present a network of interfaces. Outward transport of Na is not a feature of Ling's theory of cation transport in muscle. Such a device, however, is not applicable to the erythrocyte, where Na as well as K is actively transported and where K transport occurs from one inert phase—the plasma, across a second phase—the cell membrane, where cation movements are energized, to a third phase—the cell interior where catabolic but no anabolic activities occur. Moreover, there is in the erythrocyte no protein which could fill the role taken in muscle by myosin; stromatin, the only possible candidate constitutes but 1 % of the dried weight of the human erythrocyte. Hence, most workers in this field adhere to the view that Na and K are transported in complex combination with a carrier, presumably lipoid or lipoid soluble. Thus, if the complex XC of the carrier X with the cation C is broken down at only one face of the cell membrane, the concentration gradient for XC will carry the complex to that face, irrespective of the concentration of C on either side of the membrane and a means is afforded for the transport of C.

Flynn & Maizels (1949) originally suggested that in the maintenance of constancy of composition and volume of the erythrocyte, Na transport was the dominant factor, basing this view on the observation that in media whose composition approximates to that of plasma the manifest entry of K into the metabolizing cell never exceeds, and is often less than, the corresponding exit of Na; that when Na efflux is limited by experimental conditions K influx also falls; and that any cell containing non-penetrating anions such as protein and organic phosphate and unprotected by an external or internal resistant structure, must inevitably rupture unless a Na-excretion mechanism exists: the need of a device for the active uptake of K is not inherent. However, in view of the fact that active K influx as well as active

Na efflux must occur, Harris & Maizels (1952), while retaining the idea of the dominance of Na transport, suggested that 'inward transport of K is "geared" to outward Na transport by the use of a common carrier'. If this be so, the gear ratio must equal 1/2 since about 1·6 m.equiv. K/l. hr.$^{-1}$ are transported into the cells for about 3·2 m.equiv. Na transported out.

The apparent reciprocity between Na and K transport is further shown by the observations of Flynn & Maizels (1949) that lack of K in a medium containing suspended cells decreased the output of Na by the cells they attributed this effect to an increased passive influx of Na resulting from an insufficiency of K in the external medium to satisfy the physical requirements of the system: it was also suggested that the output of Na 'is in some way potentiated by the presence of K in the plasma and that in the theoretical but hitherto unattained K-free medium, Na efflux would cease altogether'. So, too, Hodgkin & Keynes (1953) remark that lack of K in a medium containing axons of *Sepia* reduces the efflux of Na and suggest that 'there may be a coupling between K influx and Na outflux'.

Harris & Maizels (1952), in their further discussion of transport in erythrocytes, continue: 'If the turn-over rate of K carrying groups were sufficiently low, this rate and not the external K concentration would become the rate-determining factor. Moreover, if the same groups carried Na out of the erythrocytes, the occurrence of a high Na efflux by bringing more groups to the exterior, could facilitate the increased K influx.' This theory is compatible with many of the known facts of cation transport. Thus, during cold-storage the Donnan asymmetry and the greater speed of passive Na movements compared with those of K would increase cell base and volume, while subsequent incubation in glucose containing media would restore active transport and with a K/Na ratio of $\frac{1}{2}$ would cause Na loss to exceed K gain and so lead to shrinking of the cell, the change being opposed to some extent by the Donnan asymmetry. Moreover, increased Na efflux from the Na-rich stored cell, by bringing more K-carrying groups to the exterior, would hasten K entry and cause the rate of influx to rise above that characteristic of the normal cell. So, too, if a cell is depleted of Na during cold-storage, efflux during incubation will be small, fewer carriers will be brought to the external face of the cell membrane and K influx will fail. On the other hand, one must presume that with cells in a K-free medium, return of carriers from the outer to the inner face of the cell membrane is restricted, and this may be one factor in the decreased Na efflux from cells suspended in media with very low K concentrations.

Again, if the turn-over rate of K carriers were sufficiently slow, the failure of K influx to alter appreciably when the external K concentration ($[K]_e$) was raised from 5 to 15 m.equiv./l. would be explained. But such an explanation could hardly apply to systems where $[K]_e$ had been raised to 75 m.equiv./l., for with active influx unaltered, passive influx now becomes significant and should raise the total influx to about 2·6 m.equiv./l. cells hr.$^{-1}$. E. J. Harris (this symposium) explains the observed depression of the active component of K influx when $[K]_e$ is raised, by an ingenious use of the theory of linked carriage. He observes that when $[K]_e$ is raised from 5 to 75 m.equiv./l. $[Na]_e$ is necessarily halved so that at equilibrium Na influx and hence efflux must also be halved. Applying Harris's explanation to existing data leads to the conclusion that the active component of K influx must fall from 1·6 to 0·8 m.equiv./l. cells hr.$^{-1}$, which with a passive component of K influx at 1·1 gives a total influx of 1·9 m.equiv. hr.$^{-1}$, sufficient to raise cell K by about 6 m.equiv. in 24 hr. The suggestion that active K influx decreases, while passive and total K influx increase with rise of $[K]_e$ throws doubt on the experiments of Raker *et al.* (1950), Sheppard *et al.* (1951) and of Solomon (1952) which seem to show that total K influx is independent of $[K]_e$. On the other hand, it does not wholely agree with the findings in the present paper where cells in a medium containing 75 m.equiv./l. K show a net increase of K content (corrected for extra K adsorbed in the K-rich medium and for uptake of K associated with a falling cell Na) of about 13 m.equiv./l. cells: this corresponds to a depression of the active component of influx by about 25 and not by 50%; though there is an increase in total influx of 40%. When $[Na]_e$ is halved by the addition of Li instead of K, the active component of influx falls by only 15%, and since $[Na]_i$ is halved at the same time, the K/Na gear ratio must rise by about 50% when half of $[Na]_e$ is replaced by K and by about 75% when Li is the replacing cation.

It is possible that the small increase in K influx with rise of $[K]_e$ recorded by E. J. Harris arises from his assumption that changes in cell volume are negligible. Experimentally (Table 6), if 100 ml. cells are transferred from a medium containing 2 to one with 75 m.equiv. K/l. they gain 5 ml. water and this will correspond to an additional gain of about 7 m.equiv. K of which Harris takes no account. His figures corrected for changes in cell volume correspond to a gain not of 6 but of 13 m.equiv. in 24 hr.—a figure which agrees closely with the corrected figures derived from Table 5: this, as has been seen, corresponds to a depression in true active influx not of 50 but of 25%.

Thus, the theory of linked Na-K carriage, though explaining most of the phenomena associated with cation transport in erythrocytes, does not agree with the observed effects on K influx of halving the value of $[Na]_e$, either by adding K of Li to the suspending medium and an alternative theory is set out below.

Evidence has already been put forward to show that Na and K traverse a lipoid phase of the cell membrane and it has been presumed that active transport occurs by means of carriers. However, no simple theory suffices to explain all the relevant facts of transport. Thus the existence of the complex XNa and YK respectively broken down at the outer and inner faces of the cell membrane would not explain why K influx rises when Na efflux increases, nor why active K influx falls when $[K]_e$ is raised. Hence, a more complex theory is advanced involving several assumptions. (1) It is suggested that the cell membrane contains two sorts of separated transmitting zones, both lipoid: the passive zones, through which Na and K move with the gradients, the active zones through which movements occur only by means of carriers, Na and K being transported in opposite directions against gradients of concentration and potential. (2) The amount of K adsorbed at the external phase boundary of the active zone increases rapidly as the external concentration rises from 0 to a certain critical low level (1–2 m.equiv./l.) above which level further increase in the external concentration of K is accompanied by little further increase in the amount of K adsorbed. (3) Over the rest of the external phase boundary and over the whole of the internal phase boundary of the cell membrane K adsorbed is more closely related to the concentration in the adjacent aqueous phase: a similar relation is thought to hold for Na over the whole of both faces of the cell membrane. (4) There are in the active zones common carriers for Na and K, their number being in excess of Na and K to be carried. (5) Na is liberated from the Na-carrier complex by specific enzymes related to the external phase boundary, while K is liberated from the K-carrier complex by specific enzymes related to the internal phase boundary. (6) The Na and K carriers at the respective transporting surfaces are in equilibrium with Na and K adsorbed from the adjacent watery phases, much as a citrate-calcium complex or a protein calcium complex in solution is in equilibrium with ionized calcium. In short, the active zones are envisaged as isolated lipoid cylinders containing no cations but Na and K complexes: they are thought to be bounded by active surfaces where the liberation of cations from the corresponding complex at the lipoid side of the interface is governed by the amount of the same cation which is adsorbed at the watery side of the interface, the latter opposing to some extent the energizing effect of the underlying metabolic process. The number of assumptions is somewhat large, but none are unlikely. Thus, the second, involving strong adsorption of K at low external concentrations, with little increase as $[K]_e$ rises above 2 m.equiv./l., is supported by known facts: it will be recalled that with fresh cells, $[K]_e$ at 2 m.equiv. loads half as many carriers as does Na with an internal concentration of 15 m.equiv., so that the association of K with the external phase boundary seems to be about four times as great as that of Na at the internal phase boundary; moreover, in stored cells uptake of K remains maximal with $[K]_e$ at 2 m.equiv., although $[Na]_i$ may equal 80 m.equiv. and under these conditions K at the external face must be 20 times as effective as Na at the internal face, in its ability to load carriers. If then, this theory be accepted, it becomes possible to explain many of the phenomena previously listed as characteristic of transport in the human erythrocyte. The greater speed of passive Na transfer over passive K transfer accounts for increase in cell Na, total base and volume during cold-storage, while reversal of these changes during incubation with glucose arises from the rate of Na transport exceeding that of K transport. Again, the conditions envisaged are compatible with constancy of the ratio $[Na]_i/[Na]_e$. So, too, as $[K]_e$ falls below the critical level, K adsorbed will fall sharply, the loading of K carriers will fail and K influx will fall: K de-adsorbed from the external face of the cell membrane will be replaced by Na, whose increased adsorption there will hinder the breakdown of outgoing Na-carrier complexes with a corresponding decrease of Na efflux. As $[K]_e$ rises above the critical level, it is assumed that K adsorbed at the external face of the active zones becomes maximal; thereafter K transported across the active zones remains constant, but K penetrating through the passive zones will increase with rise of $[K]_e$ and the resulting rise of $[K]_i$ will hinder the breakdown of K carriers at the inner phase boundary of the active zones and so cause the observed decrease in active influx which accompanies a rise in $[K]_e$. The fall in K influx which accompanies values of $[Na]_i$ so low as to preclude much Na efflux during incubation is more resistant of explanation: possibly, however, the Li which enters passively from the Li-rich Na-poor external medium, displacing cell Na, may be less strongly adsorbed at the inner phase boundary than Na, in which case there would be increased adsorption of the competing K within the cell and this according to the theory would lead to a decrease in active K influx. Finally, during cold-storage $[Na]_i$ rises and $[K]_i$ falls; during the subsequent incubation, the initially high $[Na]_i$ will increase the rate at which Na carriers are loaded, while the low level of $[K]_i$ will facilitate the breakdown of K carrier complexes. It follows that the increases of Na efflux and K influx observed when stored blood is incubated, are not linked but parallel events.

If all this be correct, then the apparent linkage between Na and K transport really arises from the fact that the osmotic and electrical requirements of cells and plasma are such that Na and K in the cell phase and also in the plasma phase are necessarily complementary. Thus, when cells are incubated in a medium rich in K, external Na must be reduced if isotonicity is to be maintained, while during cold-storage the initially high levels of cell K and plasma Na lead to a fall in cell K and to a complementary rise in cell Na.

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LINKAGE OF SODIUM- AND POTASSIUM-ACTIVE TRANSPORT IN HUMAN ERYTHROCYTES

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I. INTRODUCTION

The application of tracers has enabled a considerable amount of information to be amassed about the rates of penetration of various ions into living cells. Amongst these the human erythrocyte has been extensively studied, and the purpose of this contribution is to show that by a simple assumption the kinetics of movement of the alkali ions can be interpreted coherently and quantitatively for a number of different experimental conditions. That some similar assumption may be applicable to certain other cells, in particular to the erythrocytes of other species, is possible, but experimental evidence is not nearly so profuse and a stringent test is difficult to make.

The passive penetration of a membrane will normally depend upon the size and charge of the penetrating particle, and on the structure and charge of the membrane. In the following it is supposed that, for *passive* penetration taking place as a result of thermal agitation, the respective ions have different probabilities of crossing the membrane per collision made with it; and it is assumed that, in addition, an active process potentiated by metabolic reactions causes an outward flux of Na ions and an inward flux of K ions so linked that the inward active K flux is numerically related to the outward active Na flux. This might happen if the K carriers were formed chemically from Na carriers when the latter reach the outer surface of the cell.

The qualitative facts which lead to this hypothesis will be briefly mentioned, and then a step-by-step examination of the experimental data will be made in the light of it. The data have been drawn either from recent literature or from experiments made recently by the writer in collaboration with M. Maizels and T. A. J. Prankerd.

Under a variety of conditions the sum of Na and K in the cell undergoes rather little change. If fresh cells are stored in the cold, or if metabolism is stopped, an exchange of internal K for external Na proceeds, together with some net gain of Na, so that total base per cell increases. The inference drawn from this is that the rate of passive movement of Na is greater than that of K, and, as the respective concentration gradients tending to lead to K loss and Na gain are not very different, one may further conclude that the rate constant determining passive Na movement exceeds that determining passive K movement.

When cold-stored cells, which have high Na and low K content, are incubated they lose Na and gain K, but not equivalent to the Na lost, so some diminution of volume and total base also takes place. Thus the rate of active expulsion of Na against an electrochemical potential gradient is somewhat more than the rate of active accumulation of K. That the active fluxes of Na and K are not independent but are linked in some way is suggested by the following: (*a*) K influx is high into cells which (on account of high Na content) are expelling a high flux of Na; this can be deduced from results given by Maizels (1951) and Ponder (1950), though these authors do not specifically draw this conclusion; (*b*) sufficient reduction of external K reduces Na efflux (Harris & Maizels, 1952) and does not conflict with the fact; (*c*) the K influx, provided a certain minimal K concentration is present, does not vary with external concentration (Raker, Taylor, Weller & Hastings, 1950; Sheppard & Martin, 1950; Solomon, 1952). This suggests that the ingoing K is drawn from an adsorbed layer which is saturated when $K_e \geqslant c.$ 2 mmol./l. That the coupling is not a simple consequence of the Na extrusion setting up a sufficient potential difference to bring about a purely physical attraction of the K is shown by examination of the distribution of other penetrating ions, e.g. Cl or H (Sheppard, 1951; Davson, 1951; Harris & Maizels, 1952).

II. MOVEMENT OF Na

The movement of Na into the cell does not require a supply of energy because it takes place down an electrochemical potential gradient, to which the small electrical contribution is determined by pH. The rate of entry of Na seems to be proportional to its concentration between 150 and 75 m.equiv./l., if the external osmotic pressure is maintained by substitution of KCl, choline chloride, or sugar. When still more NaCl is replaced by sugar the cells lose Cl^- and gain OH^- (Davson, 1939), and this makes invalid a comparison of rates of movement of ions because pH affects the rate constants. However, over the range mentioned, and in tracer experiments in which total Na remains constant with only an exchange of ^{24}Na for ^{23}Na, one can set Na influx $= fk_1[Na_e]$ into unit volume of cell water, in which k_1 is the first-order rate constant which would hold if no electrical asymmetry were present, $[Na_e]$ is the external Na concentration, and f^2 is equal to $[Cl_e]/[Cl_i] = [H_i]/[H_e]$ and depends upon pH (the Cl ratio as function of pH is plotted in Harris & Maizels, 1952). The factor f allows for the effect of the electrical asymmetry (see Appendix).

Movement of Na from the cell is assumed to take place by both passive and active processes. The passive process, analogous to the Na entry, accounts for an efflux $k_1[\mathrm{Na_i}]/f$ from unit volume of cell water, the factor f appearing here in the denominator (cf. Appendix, p. 241). In addition, an active process, with a rate constant k_2', operates to expel a flux $k_2'[\mathrm{Na_i}]$. In each case $[\mathrm{Na_i}]$ is expressed as concentration in cell water. The total Na efflux is given by

$$\mathrm{Na\ efflux} = \left(\frac{k_1}{f} + k_2'\right)[\mathrm{Na_i}].$$

The rate of change of internal Na, allowing for the possible variation of cell volume (cf. Harris & Maizels, 1952) is

$$\frac{dV[\mathrm{Na_i}]}{V_0 dt} = fk_1[\mathrm{Na_e}] - \left(\frac{k_1}{f} + k_2'\right)[\mathrm{Na_i}], \tag{1}$$

where V_0 is cell volume at $t=0$ and V is cell volume. Provided V remains sufficiently constant the equation can be integrated, and a useful form, holding for $[\mathrm{Na_e}]$ constant and initial $[\mathrm{Na_i}] = [\mathrm{Na_i}](0)$, is

$$\exp(-k_2 t) = \frac{fk_1[\mathrm{Na_e}] - k_2[\mathrm{Na_i}]}{fk_1[\mathrm{Na_e}] - k_2[\mathrm{Na_i}](0)}, \tag{2}$$

using $k_2 \equiv \frac{k_1}{f} + k_2'$ for brevity. This equation was used to find k_2 by Harris & Maizels, who did not, however, separate k_2 into the active and passive components.

Energy requirements of Na *efflux*

At a given temperature the energy required for Na extrusion depends upon the efflux and on the logarithm of the ratio of the external to internal Na concentration, with allowance for the electrical asymmetry. In addition, if one accepts the linkage between Na and K transport, a further term appears involving the logarithm of the ratio of internal to external K concentration. This last term is multiplied by a factor depending upon the ratio of the number of K's carried in to Na's carried out. When one K is carried in for each two Na's carried out, as seems to be the case (as shown later), the expression

$$k_2[\mathrm{Na_i}]\, RT \ln\left(\frac{f\mathrm{Na_e}}{\mathrm{Na_i}}\sqrt{\frac{\mathrm{K_i}}{\mathrm{K_e}}}\right)$$

gives the Na and K movement energy requirement in unit time of unit volume of cell fluid. The value of the expression is greater when $[\mathrm{Na_i}]$ is high than when it is low. Therefore the energy demand of the Na extrusion process is more when the cells have a high Na content than when they have expelled so much that a steady, low, Na level has been reached. An example of three states during incubation of cold-stored cells is given in Table 1.

Similar figures are obtained even if the link between K and Na transport differs from that assumed in the calculation.

Table 1. *Relative rates of energy consumption and values of energy required per* Na *ion at different stages during incubation of cold-stored cells*

External Na 150 mmol./l. External K = 4 mmol./l. $f = 1\cdot2$ (pH 7·1).

$[Na_i]$ m.equiv./l.	$[K_i]$ m.equiv./l.	Relative Na efflux ions/unit time	Energy/Na ion (for 2 Na out, coupled to 1 K in)	Relative rate of energy consumption
100	50	$100k_2$	$1\cdot77kT$	$177k_2kT$/unit time
50	100	$50k_2$	$2\cdot81kT$	$140\cdot5k_2kT$/unit time
10	140	$10k_2$	$4\cdot60kT$	$46k_2kT$/unit time

k = Boltzmann's constant.

The fact that the extrusion process, although needing less energy, requires it at an increasingly high electrochemical potential evidently sets the lowest limit attained by the concentration of internal ionized Na. The extrusion process becomes decreasingly efficient as the energy requirement per mol Na expelled approaches the free energy of the potentiating reaction, and this will set a limit down to which the ratio $[Na_i]/[Na_e]$ can be driven. This fact could explain the observation of Harris & Maizels (1951) that, although the transfer constant k_2 varies widely, the ratio $[Na_i]/[Na_e]$ attained by the cells at 37° is comparatively constant, and even remains so if part of the external Na is replaced by Li.

It is perhaps desirable to repeat the evidence that Na efflux is first order with respect to $[Na_i]$, for another possibility would be that the flux should correspond to a constant energy requirement. Table 2 summarizes an experiment taken from Harris & Maizels (1952) showing that efflux is equal to the product of a constant and $[Na_i]$. In addition, there is the fact that similar values for k_2 are found both by tracer methods (constant Na_i) and the chemical method (variable Na_i).

Table 2. *To show that* Na *efflux varies as* Na_i

Time (hr.)	Interval (hr.)	$[Na_i]$	Mean Na_i (m.equiv./l. water)	Mean net loss (m.equiv./hr. × l. water)	Mean efflux (m.equiv./hr. × l. water)	k_2 hr.$^{-1}$
0		75	—	—	—	—
6	6	48·4	61·8	4·4	9·5	0·15
11	5	38·5	43·5	2·0	7·0	0·16
24	13	34	36·2	0·35	5·45	0·15

The mean efflux is found by adding the influx (5·1 μequiv./ml. cell water × hr.) to the mean net loss rate. The value for influx is found by equating the 24 hr. value of efflux (k_2 0·15 hr.$^{-1}$, $Na_i = [34]$) to the influx. Values, obtained by use of tracer Na, of the influx at 37° are similar.

The effect of temperature on Na *transfer*

In the discussion of the effect of temperature upon the rates of ion transfer it will be necessary to distinguish carefully between the flux and the rate constant. This is because the internal concentrations can vary; for example, $[\mathrm{Na_i}]$ increases and $[\mathrm{K_i}]$ decreases as the temperature is reduced. At the steady state of internal Na and K the Na efflux must equal Na influx, i.e. $fk_1[\mathrm{Na_e}] = k_2[\mathrm{Na_i}]$. If k_2 diminishes more rapidly than k_1 as temperature is reduced, then $\mathrm{Na_i}$ will rise to a higher value until a new balance is struck. Therefore the values of steady state flux (in or out) will vary equally with temperature, although the rate constants may vary differently. When the cells have not attained a steady state, as, for example, will be the case if they have recently been cooled, the effect of temperature on the flux will approximate to the effect it is having upon the rate constant. The well-known fact that cooling the cells causes $[\mathrm{Na_i}]$ to rise must mean that the temperature-dependence of k_2 is greater than that of k_1, or more specifically the active rate constant k_2' diminishes more rapidly than does the passive rate constant k_1 when the cells are cooled (for f is constant at a given pH).

The activation energy applying to the passive rate constant k_1 has been found to be about 14,000 cal./mole in the first hour of treatment at the lower temperature (Harris & Prankerd, unpublished).

Effect of pH *on the* Na *rate constants*

Provided the potential difference (E) across the cell membrane is small, as is the case in the human erythrocyte, the effect is to increase the inward rate constant by the factor f, and diminish the outward passive one by the same factor, where $f^2 = \exp(EF/RT)$, which is assumed to be equal to the ratio $[\mathrm{Cl_e}]/[\mathrm{Cl_i}]$ (see Appendix, p. 241). As the potential difference across the cell membrane is determined by pH (Harris & Maizels, 1952) the passive fluxes will vary with pH. In addition, if the fixed charge in the membrane

Table 3. *The rate constants* (fk_1) *determining* Na *influx at various* pH. *The* pH-*dependent factor f has been evaluated from the ratio* $\mathrm{Cl_e}/\mathrm{Cl_i} = f^2$ *taken from Harris* & *Maizels* (1952). *It appears that k_1 itself also varies with* pH

pH	Inward rate constant fk_1 hr.$^{-1}$	Ratio fk_1 acid/alk.	f^2	Ratio f acid/alk.	Ratio k_1 acid/alk.	Source of rate constants
7·53	0·0226	0·72	1·35	0·91	0·79	Solomon
7·06	0·0162		1·12			
7·36	0·0194	0·82	1·35	0·93	0·88	Solomon
7·12	0·0159		1·16			
7·4	0·013	0·77	1·29	0·89	0·87	Harris & Maizels
6·8	0·010		1·02			

(such as that discussed by Teorell, 1951) is pH-dependent, the value of k_1 itself will alter. Table 3 shows that the ratio of the quantity fk_1 at two different pH's is not equal to the ratio of the respective f's, that is to say, k_1 is also varying with pH. The change of k_1 happens to approximate to the change of f, but the figures are rather scattered.

The active rate constant k_2' is much more dependent upon pH, and seems to pass through a maximum at about pH 7·4 (Harris & Maizels (1951) make this statement about k_2, in which the greatest source of variation lies in the active component k_2', so it is valid to shift their conclusion to k_2').

III. MOVEMENT OF K

Experiment indicates that the active K influx is numerically related to the active Na efflux provided $K_e \geqslant c.$ 2 mmol./l. In addition, by study of systems lacking metabolite, it can be shown that passive movements in both directions take place, and it seems reasonable that eventually the ratio $[K_i]/[K_e]$ in such a system will become equal to the ratio $[Cl_e]/[Cl_i] = f^2$. Then, as for Na passive movement, there will be passive K influx $= fh[K_e]$ and efflux $= h[K_i]/f$, where h is the first-order rate constant holding in absence of a potential difference. The active K influx will be written as $rk_2'[Na_i]$, where r is the number of K ions carried in per Na ion carried out. This active component does not include the external K concentration as a factor, because it seems that there must be ample time for the inward-bound carrier to acquire a K ion irrespective of the K concentration provided the latter is more than about 2 mmol./l. This is accordingly an empirical formulation, but it may be supported, for example, by the demonstration that the cells carry adsorbed K, for which there is already some evidence (Maizels, 1935), and which is finding further support in recent experiments (Harris & Maizels, unpublished).

The differential equation describing movement of internal K can then be written

$$\frac{dV K_i}{V_0 dt} = h(fK_e - K_i/f) + rk_2' Na_i. \tag{3}$$

When it is sufficiently accurate to take the cell volume as a constant the equation can be integrated, and for constant K_e, $[Na_i] = Na_i(0)$ and $[K_i] = [K_i](0)$ at $t = 0$, one obtains

$$\left.\begin{aligned}[K_i] = [K_i](0) + f^2[K_e]\left[1 - \exp\left(-\frac{ht}{f}\right)\right] + \frac{rk_2'}{k_2}\Bigg\{ f^2\frac{k_1}{h}[Na_e] \\ \times\left[1 - \exp\left(-\frac{ht}{f}\right)\right] + \frac{[fk_1[Na_e] - k_2[Na_i](0)]\,[1 - \exp(-k_2 t)]}{\frac{h}{f} - k_2}\Bigg\}, \\ k_2 \equiv \frac{k_1}{f} + k_2'.\qquad\qquad\end{aligned}\right\} \tag{4}$$

The equation is interesting because it contains two exponential terms of different time constant. One of these has the rate constant characteristic of movement of Na, and the other the rate constant characteristic of passive outward movement of K. The two terms are operative when $[\mathrm{Na_i}]$ is varying. An example of this system has been provided by Ponder (1951), who examined the time course of the loss of K by cells suspended in NaCl solution. To this solution cells at first rapidly lose K, so after a short time the external solution will contain sufficient K for the equation to apply. Eventually (20 hr.) metabolite supply fails and a rapid K loss again occurs as if Na carriers, for want of the requisite chemical transformation, are operating on internal K. Ponder was able to express his curve as the sum of two exponentials having exponents (at 37°) $0{\cdot}27t$ and $0{\cdot}011t$ (t in hours), which compare with values of Na transfer constant (k_2) 0·25–0·35 $\mathrm{hr.}^{-1}$ and K exchange constant 0·016 $\mathrm{hr.}^{-1}$ found in isotope experiments. When $[\mathrm{Na_i}]$ remains steady the equation loses the more rapidly varying term, and in observations of K *exchange* using isotopic K the quantity h/f determines the rate. Substitution of the two steady-state conditions

$$\frac{h}{f}[\mathrm{K_i}] = rk_2'[\mathrm{Na_i}] + fh[\mathrm{K_e}] \quad \text{for K,} \tag{5}$$

and

$$fk_1[\mathrm{Na_e}] = \left(\frac{k_1}{f} + k_2'\right)[\mathrm{Na_i}] \quad \text{for Na,} \tag{6}$$

allows the equation for constant $[\mathrm{K_e}]$ of constant specific radioactivity to be reduced to $^*\mathrm{K_i}/\mathrm{K_i} = [1 - \exp(-ht/f)]$, where $^*\mathrm{K_i}/\mathrm{K_i}$ expresses the specific radioactivity of the internal K taking that of the external K as unity. No evidence of coupling between Na and K transport can remain under these conditions. If, however, $[\mathrm{Na_i}]$ diminishes in cells exposed to isotopic K (denoted by *K) there will be some uptake of *K with the rapid rate constant of the Na-active movement, in addition to the slow exchange, and this might invalidate comparison of the K exchange rates under different conditions. Similarly, cells containing *K will exchange it slowly for ordinary K from a non-radioactive solution, but will lose it (in exchange for Na) rapidly if conditions do not favour constancy of internal K level.

IV. NUMERICAL VALUES OF Na AND K FLUXES

At 37–38° C. the Na flux into and out of the cells has been given as 4·74 (Solomon, 1952) and 4·7 (Sheppard, Martin & Beyl, 1951) in μequiv./ml. cell water per hr. Under similar conditions and in the same units K flux is 2·51 (Raker *et al.* 1950), 2·57 (Solomon, 1952), 2·52 (Sheppard & Martin, 1950, with assumed $\mathrm{K_i} = 140$ μequiv./ml. cell water). To express the figures per ml. cell water a value of cell water equal to 65 % of the cell volume has been used.

To find r, the ratio of the active K influx to the active Na efflux it is necessary to separate active and passive components of the respective fluxes. For Na: as in the steady state at 37° $[\mathrm{Na_i}]/[\mathrm{Na_e}]$ comes down to about 0·07 the ratio $fk_1\Big/\left(\frac{k_1}{f}+k_2'\right)=0{\cdot}07$, and with $f^2=1{\cdot}28$, fk_1 comes to about $0{\cdot}08k_2'$, i.e. the active Na efflux is 0·92 × total Na efflux, or about 4·3 μequiv./ml. cell water per hr. For K ions the rate constant determining efflux h/f is 0·0165 hr.$^{-1}$ (mean of Raker *et al.* and Solomon). This figure is multiplied by $f^2(1{\cdot}28)$ to obtain fh and by $\mathrm{K_e}$ to obtain the passive component of the K influx. For $\mathrm{K_e}=4$ mmol./l. this comes to 0·08 μequiv./ml. cell fluid. Subtracting this from the mean of the values for total K influx one has: active K influx at 37° = 2·45 μequiv./ml. fluid per hr. Then

$$r=2{\cdot}45/4{\cdot}3=\mathbf{0{\cdot}56}.$$

An alternative evaluation can be made using the two steady-state equations, for since

$$\mathrm{K_i}=f^2\mathrm{K_e}+\frac{f}{h}r\,(\text{active Na flux}),$$

with active Na flux 0·92 × total Na flux, and h/f the exchange rate constant for K

$$\mathrm{K_i}=f^2\mathrm{K_e}+\frac{0{\cdot}92r\,(\text{total Na flux})}{(\text{K exchange constant})}.$$

Putting in usual values for $\mathrm{K_i}$, $\mathrm{K_e}$ and the flux and K exchange rate constant the value of r comes to **0·51** at 37°.

It is more difficult to evaluate r at other temperatures because information is less precise, and particularly it is doubtful how constant the internal levels of Na and K were in the experiments. In a recent set of isotope exchange experiments at 27·5°, for example, it was found that *K uptake and *Na loss was more rapid initially than after 3 hr. The value of r found was about 0·5.

It is interesting and suggestive to compare k_1 with h, the respective passive transfer constants (to which the permeability constants are proportional) for Na and K in absence of an electrical asymmetry. To find k_1 it is necessary to divide the inward rate constant fk_1 by $f(1{\cdot}13)$. Using figures from the work of Solomon (1952) or Sheppard *et al.* (1951), k_1 is about $0{\cdot}034/1{\cdot}13=0{\cdot}030$ hr.$^{-1}$ at 37°. (It is to be noted that Solomon's inward rate constant 0·022 hr.$^{-1}$ appears to refer to unit volume of cells, whereas in the present paper all figures refer to unit volume of cell water, taking this as 0·65 of the cell volume.) The rate constant of K exchange, h/f, is 0·0165 hr.$^{-1}$ at 37°, so h is 0·0187 hr.$^{-1}$. Therefore one has

$$\frac{\text{Passive permeability to K}}{\text{Passive permeability to Na}}=\frac{0{\cdot}0187}{0{\cdot}030}=0{\cdot}62.$$

At low temperatures, when the active processes are nearly stopped, the rate constants for Na and K movement are known. That for K entry at 4° is 0·0023 hr.$^{-1}$ (Sheppard & Martin, 1950) or 0·0016 hr.$^{-1}$ (Raker *et al.* 1950), whereas that for Na entry lies between 0·0035 and 0·0042 hr.$^{-1}$ (Harris & Maizels, 1952). If it be assumed that no active K influx is present at this low temperature the passive permeability ratio (K/Na) lies between 0·5 and 0·7, so it is probably the same as at 37°.

The fact that the passive permeability to Na ions is greater than that to K ions is strong evidence that neither ion penetrates the cell in the normally hydrated state, for Na ions in solution are larger than K ions, as shown by their lower diffusion constant. It seems likely that the mechanism of both passive and active processes is similar, involving attachment of ions to groups in the membrane which carry the ions across. The only difference between passive and active movement would then be that the former involves directed flow of K-carrying groups inward, and Na-carrying groups outward, whereas the latter is purely random, as in Ussing's (1949) exchange diffusion process.

The effect of K_e *on the exchange of* K

All investigators who have examined the effect of variation of K_e on the rate of K turn-over at 37° agree that the rate is little affected over a wide range of concentrations, which are obtained by replacing Na by K in the medium. In particular, Raker *et al.* (1950) used K_e between 2 and 74 mequiv./l.

This result has in fact been incorporated into the formulation by making active K influx independent of $[K_e]$, but it transpires that the sum active K influx + passive K influx is nearly independent of K_e, which is not directly obvious. One has

$$\text{active K flux} = rk_2'\text{Na}_i = rk_1\left(f\text{Na}_e - \frac{\text{Na}_i}{f}\right) \quad \text{(from (6))},$$

$$\text{passive K flux} = fh\text{K}_e.$$

So, $$\text{total K influx} = f(rk_1\text{Na}_e + h\text{K}_e) - \frac{r}{f}k_1\text{Na}_i.$$

As rk_1 is *c.* 0·016 hr.$^{-1}$ and h is 0·0187 hr.$^{-1}$, from the figures already mentioned, the factors multiplying Na_e and K_e are nearly equal, so the first term is nearly constant so long as the sum $(\text{K}_e + \text{Na}_e)$ is constant, and the second, negative, term is in any case small, about 5 % of the positive term at most. Thus K influx will not depend very appreciably upon K_e. Calculation indicates that from 74 m.equiv./l. the influx would be 7 % greater than from 4 m.equiv./l., which is not very easy to detect experimentally, particularly as cell volume may change.

The effect of pH *on* K *transfer*

When internal K remains steady the rate of exchange of K is determined by h/f. f varies with pH, but only from about 1·13 at pH 7·4 to 1·0 at pH 6·7. Probably h varies in the same direction as f, for pH is likely to have the same influence upon h as it has upon k_1. Therefore it is unlikely that a distinct influence of pH upon the K exchange will be observable, and this is in fact in agreement with the observations of both Raker *et al.* and Solomon. On the other hand, if K-depleted cells are incubated the uptake of K in exchange for cell Na involves the active Na rate constant k_2'. Then the time course of K net gain will depend upon pH in the same way as the rate constant determining Na extrusion. Flynn & Maizels (1949) and Ponder (1950) both find a pH optimum near 7·4 for net K gain (and pH net loss), agreeing with the optimum for the rate of Na extrusion (Harris & Maizels, 1951).

The effect of temperature on the rate of K *transfer*

The activation energy of the K transfer process has been measured by Raker *et al.* (1950), Sheppard & Martin (1950) and Solomon (1952). Figures for both inward and outward rate constants in the range 40–25° are between 13,000 and 15,000 cal./mole. A difficulty which arises in evaluating energy of activation applying to the outward rate constant is that even a very slow rate of net loss of K from the cells may considerably increase the value of the efflux rate constant above that deduced from exchange measurements (entry of *K), in which it is assumed that cell K concentration remains constant. Error also arises when cell volume is not constant and as in fact below 37° the K concentration falls (cf. Raker *et al.*, table 3), although the content remains steady (at 24·4°) because the cells also swell, the transfer constants applying to K movement at this, and lower temperatures should be evaluated graphically, bringing cell volume into the equation. An experiment made here on K entry at 37·5°, 27·5° and 18° showed that the rate constant applying to K entry at the two lower temperatures was diminishing for several hours after cooling the cells. The activation energy calculated after 3 hr. was nearly twice that calculated after 1 hr. The subject evidently requires further study, but it seems correct to state that the activation energy applying to the K *efflux* is close to that found for Na influx (about 13,000 cal./mole), which is to be expected if they are both similar processes. The activation energy applying to K influx is certainly higher than this when observations are made at lower temperatures (Sheppard & Martin, 1950), and it seems likely that it will be found that the *active* K influx has an energy of activation of some 25,000 cal./mole over the whole temperature range when account is taken of disturbing factors. Attention must be drawn to the fact that cells which are not in a steady state at the temperature in question will have time variable K fluxes, and the activation energy deduced will apply to variable proportions of active and passive processes.

It is interesting that the activation energy of the glycolytic reaction is very close to that of the passive-ion movement. This might indicate that the passive movement of some ion across the cell membrane controls glycolysis. In addition, it is possible to envisage an active transfer mechanism whose rate would depend upon the product of the concentration of a labile intermediate and the concentration of those ions having 13,000 cal./mole activation energy. The overall activation energy of the active process might then come to the sum of the energies applying respectively to passive penetration and to the rate of glycolysis.

The exchangeability of cell Na

Both Solomon (1952) and Sheppard *et al.* in two papers (1951) found that one-third to one-half of the cell sodium did not appear to participate in the exchange process *in vitro*. Analyses made recently (Harris & Maizels, unpublished) also indicate that cells suspended in Na-free solutions do not lose all their Na. The tracer experiments quoted, and others made here, indicate a variable behaviour of the cell Na, as if some external factor, operative *in vitro*, brings about the partial immobilization of part of the Na. In saline suspensions the rate constant for Na efflux diminishes after some hours storage at 37° (Harris & Prankerd, 1953). Use of plasma-saline mixtures appears to favour consistent results, but even whole bloods to which are added a very little radioactive preparation frequently show an incomplete equilibration of their Na after 24 hr. incubation. One possible explanation for this apparently incomplete exchange (though not for the slowing of the rate constant) would be provided if a part of the Na was so readily exchanged that the shortest wash, as usually applied to remove radioactive extracellular fluid, brought about a substitution of ordinary Na for the isotopically labelled Na. As mentioned when discussing K exchange there is evidence for Na and K being adsorbed, and it is found that unwashed samples of cells drawn from a suspension just after addition of tracer Na carry several times more radioactivity than that expected for the extracellular fluid alone.

The effect of fluoride

In low concentrations (2–10 m.equiv./l.) fluoride inhibits glycolysis, and after 1–2 hr., during which the cells use up reserves of metabolite, the active Na extrusion ceases (Maizels, 1951; Harris & Maizels, 1951). Na movement is then governed by the passive rate content. In high concentrations of fluoride (60 m.equiv./l.) Davson (1941) found that a drastic change in behaviour was caused in the rabbit erythrocyte. K was lost without a corresponding gain of Na, so the cells shrank. An experiment has indicated a similar behaviour on the part of human cells, which under these conditions have a Na efflux several times that of the usual passive efflux, and a high permeability to K, so it seems that a change in the membrane structure has been induced.

Other alkali ions

Some isolated observations of the effect of other alkali ions on Na and K movements have been made (Ponder, 1950; Flynn & Maizels, 1949; Solomon, 1952). Using tracer K in a mixture containing Rb, Solomon made two experiments. These suggest that, within experimental error, K influx is reduced in the ratio $K_e/(K_e+Rb_e)$. The figures are

$K_e/(K_e+Rb_e)$	$\frac{\text{K flux in presence Rb}}{\text{K flux in absence Rb}}$
0·58	0·52
0·61	0·66

This result would follow if the inward-going carriers made little distinction between the two ions. Li and Cs apparently are not taken in by the K carriers, for they did not reduce K influx directly; an indirect effect of Li is mentioned later.

Substitution of Li, K or Rb for two-thirds of the external Na reduces Na influx, Li having rather more effect than the other ions. The total influx of Li + Na is about 4·0 m.equiv./l. cell fluid per hr. as compared to the usual Na influx of 4·7 m.equiv./l. cell water per hr., which suggests that the passive rate constant applying to Li entry is lower than that applying to Na entry.

Cells exposed to mixtures of Li and Na retain their usual Na efflux rate constant (k_2) (Harris & Maizels, 1951), and ultimately the ratio Na_i/Na_e becomes equal to that found in the high Na solution. The reduction of $[Na_i]$ which occurs in the Li mixture will lead to Na efflux diminishing in proportion, and on the hypothesis advanced here this will lead in turn to a reduced influx of K, so $[K_i]$ will fall. That this reduction of K influx and K_i does occur has been observed both by Ponder (1950) and Flynn & Maizels (1949).

Other erythrocytes

Figures for the rates of turn-over of Na and K in cells of some other species have been given by Sheppard *et al.* (1951). These cells differ from those of man because they have low $[K_i]$ and high $[Na_i]$; thus they are not likely to require a very efficient K transporting system. A rough evaluation of the ratios 'r' and h/k_1, i.e. the respective active and passive permeability ratios for K ions compared with Na ions has been made:

Animal	$[\mathrm{Na_i}]$ µequiv./ml. cell water	$[\mathrm{K_i}]$ µequiv./ml. cell water	h/k_1	r
Dog	168	8	0·1	0·02
Sheep	156	15	0·1	0·01
Cow	102	37	0·3	0·3

It is remarkable that again the rate constants determining K passive movement are lower than those determining Na passive movement, and the active process carries fewer K ions than Na ions.

V. CONCLUSION

In conclusion, the results obtained for human red cells indicate that a single mechanism brings about active Na extrusion and active K accumulation. The K must be drawn from a reservoir which is filled to a level independent of the external concentration when the latter exceeds 1–2 m.equiv./l. In addition to the active process, there are passive fluxes applying to each ion operating in each direction. The rate constant determining passive sodium movement is greater than that determining passive potassium movement.

Recent observations by Steinbach (1952) on muscle and Hodgkin & Keynes on nerve (1953) suggest that in these tissues also there is a linkage between the active movement of sodium and the accumulation of potassium, so it is possible that a common chemical process operates in a number of different biological materials. The elucidation of the chemistry of this process appears to be one of the most important issues in the field of so-called permeability studies.

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APPENDIX

The effect of a small potential difference upon inward and outward rate constants

By assuming a constant field across the membrane, Goldman (1943) and later authors have derived integral forms of the diffusion equation which can be written for a univalent positive ion:

$$\text{influx} = P\frac{eV}{kT}\;\frac{\text{(outside concentration)}}{1-\exp(-eV/kT)},$$

$$\text{efflux} = P\frac{eV}{kT}\;\frac{\exp(-eV/kT)\,\text{(inside concentration)}}{1-\exp(-eV/kT)}.$$

P = permeability constant, proportional to the transfer constant, in absence of the electrical field. k = Boltzmann's constant, V the magnitude of the potential difference which is across the membrane (inside of cell negative), e = the electronic charge, T the absolute temperature.

When eV is small compared with kT, as is the case in the human erythrocyte as judged by the chloride ratio, the exponentials approximate to

$$1 - eV/kT + \tfrac{1}{2}(eV/kT)^2.$$

Substituting this one obtains

$$\text{influx} = \frac{P\text{(outside concentration)}}{1-\tfrac{1}{2}eV/kT},$$

$$\text{efflux} = \frac{P\text{(inside concentration)}}{1-\tfrac{1}{2}eV/kT}\left(1 - eV/kT + \tfrac{1}{2}(eV/kT)^2\right)$$

$$= P\text{(inside concentration)} \times (1 - \tfrac{1}{2}eV/kT), \text{ nearly.}$$

Putting $1 - \tfrac{1}{2}eV/kT = f$, it is seen that the operative inward permeability constant in presence of the field is fP, and the outward one is P/f.

Also, for a positive ion (e.g. H) which is not subject to active transport, when the concentrations are steady influx = efflux and (inside concentration) $= f^2$(outside concentration). For a negative ion, such as Cl, the equations are reversed and $\text{Cl}_e/\text{Cl}_i = f^2$.

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THE ACCUMULATION OF AMINO-ACIDS WITHIN STAPHYLOCOCCAL CELLS

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I. INTRODUCTION

Our attention was first drawn to problems of active transport in bacterial cells when, in the course of studies on the assimilation of amino-acids by bacteria (Gale, 1953), we found that certain Gram-positive bacteria contained high concentrations of free amino-acids inside the cells (Gale, 1947; Taylor, 1947), and the problem arose, among many others, of the mechanism whereby these amino-acids were retained in such high concentrations.

It is now known that many cells of microbial, plant and animal origin contain free amino-acids. Christensen, Riggs, Fischer & Palatine (1952*a*, *b*) have followed their earlier studies on the accumulation of glycine by various animal cells by a detailed investigation of amino-acid accumulation by mouse ascites tumour cells. These cells affect a marked concentration of many amino-acids, including such as α-γ-diaminobutyric acid which do not occur naturally, and it seems that some amino-acids displace potassium ions within the cells. In the case of diaminobutyric acid the potassium displacement is almost complete and results in an intense concentration of the amino-acid across the cell wall. The effect of metabolic activity of the cells on the amino-acid concentration has not been studied in detail and, although no oxidizable substrates are added in addition to the amino-acids, it is probable that the tumour cells accomplish an endogenous respiration during the concentrative action, since the addition of inhibitors such as cyanide, dinitrophenol and arsenate markedly decreases the ability to concentrate glycine (Christensen & Riggs, 1952). The accumulation of glycine is accompanied by a swelling of the cells, so it would appear that the amino-acid is osmotically active within the cells. Our own studies have concerned the accumulation of amino-acids by bacteria and yeasts, and, for the purposes of this symposium, I intend to restrict my discussion mainly to one organism, *Staphylococcus aureus*. Taylor (1947) studied the ability of a range of bacteria to accumulate amino-acids, and *Staph. aureus* strains proved to have the highest concentrating activity towards glutamic acid. Although glutamic acid may not be the amino-acid which undergoes the highest concentration across the staphylococcal cell-wall (see Table 1), its ease of estimation by the specific decarboxylase method (Gale, 1945) has resulted in a greater amount of knowledge accumulating concerning its transport than for any other amino-acid. The investigations have been reviewed elsewhere (Gale, 1949*a*, 1953), and certain aspects of them have been discussed in a previous symposium of this Society (Gale, 1948).

The concentration of the free amino-acids within staphyloccocal cells varies with the conditions holding at the time of harvesting of the cells from a growth medium. Factors which affect the concentration include the concentration of free amino-acids in the external medium, the phase of growth when harvesting occurs, the availability of sources of energy such as fermentable carbohydrates, the availability of other amino-acids, the rate of protein synthesis within the cells, and the presence of phosphate and other ions in the external medium. By a judicious selection of conditions it is possible to obtain suspensions of organisms which possess very little free amino-acid within the cells; these 'deficient' cells can then be used to investigate the conditions under which specific amino-acids will accumulate within the cells.

II. ACCUMULATION OF LYSINE AND GLUTAMIC ACID

If such deficient cells are suspended in a solution of lysine, free lysine begins to accumulate rapidly within the cells, and the accumulation continues until the internal concentration is some 4–60 times greater than that in the external solution, the concentration gradient becoming larger as the external concentration decreases. This accumulation is not affected by metabolic inhibitors such as cyanide or dinitrophenol, takes place rapidly at 2° C., and has a temperature coefficient of 1·4 which is within experimental error of the value for free diffusion. The process appears to be one of diffusion across the cell-wall followed by a distribution between internal and external media essentially similar in properties to a Donnan distribution (Gale, 1947, 1953; Najjar & Gale, 1950; McQuillen, 1950*a*).

If deficient cells are suspended in a solution of glutamic acid, even at 37° C., there is no accumulation of glutamic acid within the cells as long as precautions are taken to exclude metabolic sources of energy. If an energy substrate such as glucose is added to the incubation mixture, then rapid accumulation of free glutamic acid takes place within the cells, and the internal concentration may rise to as much as 400 times that in the external medium. The accumulation is abolished by any inhibitor which prevents the metabolism of glucose, does not take place at 2° C. or less, and has a temperature coefficient of 2·7–2·8. If the rate of internal accumulation is determined at various external concentrations, that for lysine is found to vary in an approximately linear manner with external concentration, while that for glutamic acid is independent of external concentration except for low values of the latter (Gale, 1947). Further, if the amount of amino-acid appearing within the cell is compared with that disappearing from the external medium, it is found that the transport of lysine is quantitative, whereas there is frequently a marked over-all loss during the transport of glutamic acid.

If cells are loaded with glutamic acid by incubation in the presence of glucose, then washed and resuspended in water or saline, there is a very slow loss of glutamic acid from inside the cell to the external medium. The addition of glucose stops this loss, but uncoupling agents such as sodium azide or dinitrophenol do not accelerate it.

The accumulation of glutamic acid by *Staph. aureus* is accompanied by an increase in the potassium content of the cells. The amount of potassium taken up by the cells corresponds to not more than 1 atom per molecule of glutamic acid accumulated (Davies, Folkes, Gale & Bigger, 1953). If the experiments are carried out in a medium freed from potassium as far as possible, the accumulation of glutamic acid is decreased but not abolished, the increase in amino-acid within the cells being partially balanced by an increase in sodium ions. The ability to accumulate glutamic acid is fully restored by the addition of potassium but not magnesium or ammonium ions.

The accumulation of glutamic acid thus takes place only when metabolic processes are occurring and, as a first approximation, would appear to take place as a result of active transport across the cell-wall structures.

If the accumulation process involves a metabolic link between glutamic acid and glucose breakdown, it should be possible to inhibit the enzyme or enzymes concerned in the link and so prevent glutamic acid accumulation without inhibiting glucose fermentation or respiration. In the course of our studies we have found six substances having such inhibitory actions: sodium azide, 2.4-dinitrophenol, crystal violet (Gale, 1951), 8-hydroxyquinoline (Gale, 1949*b*) and, under somewhat different circumstances, penicillin (Gale & Taylor, 1947) and bacitracin (Paine, 1951). Sodium azide and dinitrophenol have been shown to uncouple oxidative phosphorylation processes in yeast and mitochondria, and the general properties of their inhibition of glutamic acid accumulation may mean that similar coupled reactions are involved here. 8-Hydroxyquinoline appears to act by inactivation of a metal (manganese or magnesium) necessary for the accumulation process (Gale, 1949*b*). Penicillin and bacitracin prevent glutamic acid accumulation without affecting glucose metabolism if the cells are allowed to grow in the presence of either antibiotic for 30–90 min. before harvesting. It is probable that the effect in this case is a secondary one representing a loss of function following upon some more direct inhibition.

III. ACCUMULATION OF OTHER AMINO-ACIDS

Chromatographic examination of extracts from staphylococcal cells shows that many amino-acids, other than glutamic acid and lysine, exist in the free state within the cells. It would be of interest to know which of these accumulate as the result of active processes. Enzymic methods of estimation which can clearly distinguish between amino-acids inside and outside cells are available for only a few amino-acids (Gale, 1945; Krebs, 1950). Such methods show that the accumulation of glutamic acid, aspartic acid and histidine is increased by the presence of glucose. Recently, C^{14}-labelled amino-acids have become available, and it has been possible to investigate the distribution of radioactivity across the cell-wall when *Staph. aureus* is incubated with specific amino-acids with and without glucose. The organism can acquire endogenous stores of energy under some conditions of growth, and some accumulation of, for example, glutamic acid will occasionally occur in the absence of added glucose as a result of the utilization of such stores. However, this utilization can be abolished by the use of 2.4-dinitrophenol, and the method adopted has been to investigate the accumulation of radioactivity in the presence of glucose with and without an uncoupling concentration of 2.4-dinitrophenol (DNP). The measurements give the radioactivity of the external and internal media, and it does not follow that the activity of the cell contents is necessarily due to the amino-acid used alone. However, the ability of the staphylococcus to break down amino-acids is very limited (Hills, 1940), and the ratios obtained give an indication of the importance of active processes in the assimilation of the amino-acids studied. Table 1 summarizes the results obtained.

The concentration ratio for internal and external radioactivity was determined in each case and column (*c*) of Table 1 shows the increase in this ratio when DNP was omitted. In the case of glutamic acid, which has been exhaustively investigated by the enzymic method, the ratio obtained in the absence of DNP was 324, which agrees well with the value of 400 obtained by the enzymic method for low external concentrations of glutamic acid. In the presence of DNP the ratio falls to 2·8, and it is clear that active processes, which can be abolished by DNP, result in a very great increase in the accumulation of glutamic acid. In the case of lysine, the isotope experiments indicate a marked accumulation in the presence of DNP, and this accumulation increases about 3 times when DNP is omitted. It would appear that, although lysine accumulates in the absence of active processes, the accumulation increases when energy is made available. The amino-acids which resemble lysine, in that a marked accumulation occurs in the presence of DNP, are arginine, alanine and glycine. Glycine gives the highest concentration ratio under these conditions, and its accumulation would appear to be decreased by glucose metabolism. It is possible that the accumulation of glycine in *Staph. aureus* resembles that in mouse ascites tumour cells and takes place by displacement of potassium. The amino-acids which do not accumulate markedly in the presence of DNP and whose concentration is increased by active processes are proline, glutamic acid, phenylalanine, aspartic acid and methionine/valine. The internal concentration in all cases is calculated on the assumption that the whole volume of the cell is available for accumulation purposes; the true 'water-space' of the cell must be considerably less than this, and consequently the concentration gradients given are smaller than those actually occurring across the cell-wall. It would seem that some degree of accumulation of all the amino-acids must occur even in the absence of active processes.

Table 1. *Active processes involved in the accumulation of free amino-acids within* Staphylococcus aureus

Washed suspensions of cells were incubated with C^{14}-labelled amino-acids, glucose, buffered salt solution with and without 0·01 M 2·4-dinitrophenol. After 60 min. at 37° C., the cells were centrifuged down and the free amino-acids liberated, after exhaustive washing, by treatment with cetyl-trimethylammonium bromide. The radioactivities of the internal fluid and of the external medium were determined and expressed as a ratio.

Amino-acid	Concentration ratio $\frac{\text{Internal}}{\text{External}}$		Increase in concentration ratio due to active processes
	(*a*) Glucose present	(*b*) Glucose + DNP present	$c = a/b$
Proline	1670	9·7	171
Glutamic acid	324	2·8	104
Phenylalanine	16	0·9	17·2
Aspartic acid	30·7	4·24	7·2
*Methionine/valine	26·2	3·96	6·6
Threonine	13·8	7·0	1·97
Tyrosine	8·65	5·36	0·62
Lysine	196	63	3·12
Arginine	96·5	76	1·26
Alanine	19·7	19·6	1·01
Glycine	81·5	92·8	0·89

* The labelled amino-acids were obtained biosynthetically and fractionated chromatographically; valine and methionine were combined in one fraction.

IV. ACTIVE TRANSPORT OF GLUTAMIC ACID

The purpose of this symposium is to discuss the nature of the active transport process, and I intend to devote the rest of this contribution to a consideration of the active accumulation of glutamic acid in the staphylococcus. There would appear to be three possible explanations for the experimental observations:

(1) That the cell-wall structures include a membrane impermeable to glutamic acid and that, during the breakdown of glucose, some linked metabolism occurs which results in the production of a derivative of glutamic acid which can diffuse through this membrane and is reconverted to glutamic acid within the cell.

(2) That the cell-wall structures are impermeable to glutamic acid but contain a substance which is capable, during glucose metabolism, of combining with glutamic acid and acting as a carrier across the impermeable barrier.

(3) That the cell-wall structures are freely permeable to glutamic acid, but that, when glucose is metabolized within the cell, a derivative of glutamic acid is formed which is unable to diffuse out of the cell and consequently accumulates. This derivative would be estimated as free glutamic acid in the experimental procedures so far used.

A fundamental difference between hypotheses (1) and (3) is that, in the former, coupled metabolism must take place near the surface of the cell, outside the permeability barrier, whereas, in the latter, the metabolism occurs within the cell. If the reaction is on the surface of the cell it should be possible to reproduce the reaction by supplying breakdown products of glucose. We immediately think of the possibility of generating energy-rich bonds probably involving phosphate; this would give a system analogous to that postulated by Gourley (1952) for the transport of phosphate in the erythrocyte. However, it has not been possible to promote glutamic acid transport by the addition of hexose- or triosephosphate, adenosinetriphosphate, or metaphosphates. Negative results of this nature can always be explained away by the further postulate that the site of the coupled metabolism is not actually on the surface of the cell but lies beneath the surface while being above the barrier to glutamic acid penetration. Cytologists will not necessarily quarrel with a postulate which endows the bacterial cell with a series of outer coats.

Serious consideration has been given to the possibility that glutamine might be the form in which glutamic acid passes across the cell-wall (Mitchell, 1949). The synthesis of glutamine from glutamic acid requires the intervention of adenosinetriphosphate (Elliott, 1951), and an attractive hypothesis can be built around the idea that coupled phosphorylation near the surface of the cell results in synthesis of glutamine, which diffuses through the cell-wall and undergoes irreversible hydrolysis to glutamic acid within the cell. However, addition of glutamine to suspensions of staphylococci does not result in the accumulation of glutamic acid within the cells unless glucose is also added. Further, the addition of α-amino-γ-methylsulphinylbutyric acid does not affect the accumulation of glutamic acid within *Staph. aureus*, although it acts as an inhibitor of the system converting glutamic acid to glutamine (Waelsch, Owades, Miller & Borek, 1946; Elliott & Gale, 1948) and affects the assimilation processes for glutamic acid in *Strep. faecalis* (Gale, 1949*a*).

If a metabolic modification of glutamic acid occurs prior to its passage into the cell, it should be possible to obtain some indication of the nature of this modification by a study of the accumulation of glutamic acid within cells presented with derivatives of that amino-acid. A number of glutamic acid derivatives have been tested in this sense both in the presence and absence of glucose. Of the substances tested, only two have been found which will give rise to glutamic acid inside the cell when incubation takes place in the absence of glucose: *N*-phosphoryl-glutamic acid and the diethyl ester of glutamic acid. *N*-phosphoryl-glutamic acid is relatively unstable and decomposes to release glutamic acid, but addition of the substance to washed suspensions of *Staph. aureus* is followed by the appearance of free glutamic acid within the cells at a rate which varies widely but has been as high as 75 % of that obtained in the control incubated with glucose and glutamic acid. The temperature coefficient for the over-all process is 2·4, and it is possible that phosphoryl-glutamic acid may be acting as an energy source rather than entering the cell unchanged. In the presence of glucose, the internal accumulation of glutamic acid occurs at the same rate whether the external source is glutamic acid or the *N*-phosphoryl derivative. The diethyl ester of glutamic acid gives rise to free glutamic acid within the cells at a slow rate with a low temperature coefficient (1·8). The esterase activity of these cells is low, and the slow rate of accumulation of free glutamic acid within the cells is probably due more to a slow rate of de-esterification than to a slow penetration. No evidence could be obtained for esterification of glutamic acid by the cells.

A number of simple peptides containing glutamic acid have also been tested (see Table 2), but in no case has free glutamic acid appeared within cells incubated in the absence of glucose. In the presence of glucose, some peptides, e.g. α-glutamyl-valine or α-glutamyl-leucine, give rise to glutamic acid within the cells more rapidly than does glutamic acid itself as external source. α-Glutamyl-glutamic acid or α-glutaminyl-glutamic acid give rise to free glutamic acid within the cells but at a slower rate than glutamic acid itself. There is a suggestion that the effectiveness of the external source may be related to its lipoid solubility, but further investigations are needed with a wider range of peptide structures.

Table 2. *Glutamic acid derivatives giving rise to free glutamic acid within* Staphylococcus aureus

	Comparative rate	Temperature coefficient 20–30° C.
A. Free glutamic acid obtained only in presence of glucose		
α-L-Glutamyl-L-leucine	146	2·33
α-L-Glutamyl-L-valine	140	2·25
L-*Glutamic acid*	*100*	*2·8*
α-L-Glutamyl-glycine	88	—
α-L-Glutaminyl-L-glutamic acid	69	2·5
L-Glutamine	60	2·4
α-L-Glutamyl-L-glutamic acid	45	2·0
Glycyl-L-glutamic acid	22	—
γ-L-Glutamyl-L-valine	20	—
γ-L-Glutamyl-L-leucine	16	—
Glutathione	15	2·5
γ-L-Glutamyl-L-glutamic acid	13	2·4
γ-L-Glutamyl-L-aspartic acid	5	—
γ-L-Glutamyl-glycine	3	—
B. Free glutamic acid obtained in absence of glucose		
N-phosphoryl-L-glutamic acid	40–75	2·45
Diethyl-L-glutamic ester	10–35	1·8

Comparative rate gives the rate of appearance of free glutamic acid within the cells incubated at 37° C. compared with the rate when glutamic acid and glucose form the external source.

Rothstein & Meier (1948), using isotopic methods, demonstrated the presence of phosphatases at the surface of yeast cells, while Barron, Muntz & Gasvoda (1948) showed that glucose oxidation by yeast could be inhibited by uranium ions and the inhibition released by addition of phosphate. Since cell-free enzyme preparations were not affected in the same way, the suggestion was put forward that the uranium was acting by combination with proteins on the cell surface which were responsible for the oxidative processes. McQuillen (1950*b*) has shown that the staphylococcal cell will adsorb uranium ions, and we have further found that saturation of the cell surface with uranium will prevent the accumulation of glutamic acid within the cell. Again, the inhibition can be reversed by washing the cells in phosphate buffer. These findings are consistent with the hypothesis that factors on the surface of the cell play a role in the over-all accumulation process.

Free penetration: active accumulation

We can now turn our attention to the third hypothesis which postulates that glutamic acid penetrates the cell-wall structures and that a non-diffusible derivative is formed inside the cell. First we must consider the grounds on which the amino-acid within the cell is called 'free glutamic acid'. In our studies at Cambridge we have estimated glutamic acid by the specific decarboxylase method (Gale, 1945). Glutamic decarboxylase will attack the L-isomer of glutamic acid only, and requires that both carboxyl-groups and the amino group are unsubstituted (Gale, 1946). The enzyme will not attack any peptide of glutamic acid. Glutamine and *N*-phosphoryl-glutamic acid are attacked only after the substituting groups have been removed; the crude preparations of decarboxylase contain enzymes which will carry out these removals. The material which is liberated from within the staphylococcal cell is attacked by the enzyme at exactly the same rate as glutamic acid. Since the enzyme cannot penetrate the intact cell, it is only possible to test the action of the enzyme on the intracellular material after its release from the cells, and the necessity for rupturing or altering the cell structure introduces doubt concerning the identity of the material investigated and the material as it exists in the untreated living cell. Methods that have been used to release the internal material are

(1) subjecting the cells to 100° C. for 10–15 min.;

(2) shaking the cells with small glass beads at 50 vibrations/sec. for 15–20 min. in the cold;

(3) subjecting the cells to supersonic vibration (20,000 cyc./sec.) for 5–10 min. in an ice-cooled vessel;

(4) treating the cells with acetone-ether mixtures or with 5 % trichlor-acetic acid;

(5) treating the cells with bactericidal concentrations of tyrocidin or detergent substances such as cetyl-trimethyl-ammonium bromide;

(6) crushing the cells under pressure at −15° C. in the Hughes press (Hughes, 1951).

In all cases material is obtained which contains glutamic acid when tested with the specific decarboxylase or by chromatographic procedures. Nevertheless, it is clear that many of these treatments are drastic and might well cause decomposition of labile derivatives either as a result of heating or of the liberation of catabolic enzymes. Even the material obtained from method 6 must be thawed before it can be tested. Although no evidence has been obtained that the internal glutamic acid is other than free glutamic acid there can be no confidence that some alteration in its nature has not occurred at some stage in the procedure.

Cowie, Roberts & Roberts (1949), Roberts, Roberts & Cowie (1949) and Roberts & Roberts (1950) investigated the somewhat similar situation that arises when potassium accumulates within *Escherichia coli* during glucose metabolism. The cells do not accumulate potassium in the absence of glucose breakdown, and the process has the appearance of involving active transport. By the use of isotopically labelled metals, these authors showed that free diffusion of both Na and K ions occurs across the bacterial cell-wall, and that rapid equilibration occurs between the external medium and the 'water space' within the cells. From the sodium distribution they calculated that the water space occupied approximately 70% of the cell volume. When the cells were actively metabolizing glucose, the uptake of potassium increased and the accumulated potassium appeared to be held in a non-diffusible form within the cells. It was suggested that potassium combined with breakdown products of glucose to produce the non-diffusible material. The accumulation was therefore not due to active transport of potassium across an impermeable barrier but to the formation of a non-diffusible metabolic product within the cell, free diffusion of potassium ions still occurring between the internal and external media. The situation therefore corresponds to hypothesis 3.

The experimental findings concerning glutamic acid accumulations are superficially very similar to those found for potassium accumulation in *Esch. coli*, so Britten (1952, 1953) undertook an investigation with *Staphylococcus aureus* using ^{14}C-labelled glutamic acid. In the first place he found free exchange between glutamic acid of the 'water space' in the cells and of the external medium whether metabolism of glucose was occurring or not. Over and above this exchange, however, he found that, when active metabolism was occurring, glutamic acid became stored within the cells in a form which was less readily exchangeable with the external medium. If the temperature was dropped to 4° C., exchange between external glutamic acid and 'stored glutamic acid' fell to insignificant values. It was further found that, if the cells were incubated with glutamic acid and then removed from solution, the amount of 'stored glutamic acid' continued to increase at the expense of the glutamic acid in the water-space of the cells. The picture that develops from these investigations is that glutamic acid passes freely into the water space of the cells and, when glucose metabolism occurs, enters into some metabolic process which results in the formation of a non-diffusible form, this non-diffusible material constituting the 'free glutamic acid' of the previous studies. Britten was again unable to distinguish between the 'stored glutamic acid' after liberation from the cell and free glutamic acid, but, unless the interpretation of this type of experiment is wrong, there would appear to be no escape from the conclusion that the internal glutamic acid cannot be *free* glutamic acid. It may be some highly labile derivative or possibly it is combined in some labile manner with the cell proteins.

We know little or nothing of the interior organization of the bacterial cell. A botanist presented with a situation similar to that discussed here might suggest that free diffusion of potassium and glutamic acid occurs into the internal medium of the cell and that, when glucose is available, active transport into, and accumulation within, a vacuole takes place. I do not know whether vacuoles exist inside the staphylococcus. Britten calculates that the water-space of the staphylococcus occupies 35–45% of the cell volume, so there may be room for a small vacuole in the rest of the cell. If there is, then our problem is merely removed from the door-step to the serving-hatch but, for the present, there would seem little point in postulating a hole in a bacterium to explain a hole in a postulate.

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TRANSPORT OF PHOSPHATE THROUGH AN OSMOTIC BARRIER

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Much of the present-day knowledge of biochemistry is centred about the part played by phosphate in coupling thermodynamically natural with thermodynamically unnatural processes in living organisms. Nevertheless, very little is known of the mechanism of transfer of phosphate molecules through the membranes which form the connecting links between biological media. The view, expressed nicely by Rosenberg (1948), that by virtue of specific permeability properties, the natural membranes act as connecting links between particular components of the phases which they separate has its counterpart in the view of the enzymes as couplers of reactions which can proceed only on or in the enzyme molecules. Rosenberg's treatment shows, in fact, that the energetics of the reactions in two phases connected by a membrane can be described in the same terms as 'homogeneous' enzyme-linked reactions; the important implication being that the efficiency (or reversibility) of transport reactions is determined by the specificity of membrane permeability, exactly as the efficiency of coupled enzyme reactions is determined by the enzyme-substrate and enzyme-carrier specificities. This merging of the terms of description has appropriately coincided with the realization that the permeability properties of membranes to the substrates which they transport may be dominated by enzymic specificities. In complex biochemical systems, such as those carrying out oxidative phosphorylation (e.g. Slater & Cleland, 1953), the osmotic and enzymic specificities appear to be equally important and may be practically synonymous.

Although the study of membrane-transport phenomena in combination with active metabolism has been responsible for great advances in the outlook upon permeability problems, it is unfortunate that the experimental systems in which active transport can be studied should, in general, contain so many variables. This would seem to be particularly true in the case of phosphate transport, since the phosphate molecules may be involved at several points in the train of energy-yielding reactions required to drive the active transport. There is no *a priori* reason to suppose, however, that the transport reaction may not be studied when isolated from the energy-yielding reactions. Ussing (1947) has proposed to account for the passive one-to-one exchange of cations across a membrane by a process, referred to as exchange-diffusion, in which the ions are supposed to saturate a carrier, in combination with which they pass across the membrane which they cannot traverse in the free state. In 1950, Roberts & Roberts, using ^{32}P, found that a one-to-one exchange of inorganic phosphate molecules occurred across the cell membrane of resting *Bacterium coli.* They maintained, however, that the so-called inorganic phosphate of *Bact. coli* (extracted in dilute trichloroacetic acid) was not free, but was adsorbed on a fixed number of acid-labile sites within the cells, the membrane of which they considered to be freely permeable to phosphate ions. The one-to-one phosphate exchange was therefore described by the self-explanatory term exchange-adsorption. Roberts & Roberts (1950) were not alone in suggesting that the so-called internal inorganic phosphate might be fixed in an adsorption complex within the cells. Similar suggestions were, for instance, made by Kamen & Spiegelman (1948) working on baker's yeast, Green, Atchley, Nordman & Tepley (1949) working on the cyclophorase system of mamalian tissues, and Harman (1950) working on mammalian heart-muscle mitochondria.

An exchange of inorganic phosphate across the cell surface, apparently similar to that reported for *Bact. coli*, was observed in a *Staphylococcus* by Mitchell & Moyle (1953). The rate of the exchange in resting cells suspended in a balanced salt medium containing 10 mM-phosphate at neutral pH was 1·4 μmole/g. cell dry wt./min. at 25° C. The reciprocity of the exchange was extremely strict when precautions were taken to minimize residual respiration (Mitchell, 1953*a*), the net flux being much less than 1 % of the exchange flux. When, however, the cells were allowed to respire in the presence of glucose, for which they possess powerful oxidative systems, a net flux of phosphate was directed inward at the rate of 1·4 μmole/g./min. as before, and the outflux fell virtually to zero (Mitchell & Moyle, 1953). We therefore considered it probable that the same transport mechanism was employed in both the uptake and the exchange reactions. This being the case, it occurred to us that a study of the latter reaction might yield information about the transfer process because of the elimination of the large number of variables connected with the energy-yielding reactions. It was necessary, however, to postpone any study of the exchange reaction *per se* until it could be decided whether the uptake of inorganic phosphate represented a true active transport of phosphate across an osmotic barrier impermeable to phosphate ions, or whether it represented an adsorption process like that considered by Roberts & Roberts (1950).

Two kinds of experimental approach towards a decision between the alternative exchange hypotheses were adopted. The first was to determine whether or not an osmotic barrier impermeable to phosphate ions exists near the cell surface. This could be done by determining the degree of dilution of a known addition of phosphate to a thick cell suspension and estimating the part of the volume of the suspension accessible to the externally added phosphate. If a phosphate-impermeable barrier were to exist near the external surface of the staphylococci, the effective cell volume should be approximately equal to the volume of a close-packed centrifuged pad less 26%, the interspace volume for close-packed spheres (Conway & Downey, 1950). If, on the other hand, there were no osmotic barrier for phosphate, or if the barrier were broken by reagents such as trichloroacetic acid or butanol, the effective cell volume should be approximately equal to the apparent specific volume of the materials of the cell, namely, *c.* 0·8 ml./g. The effective phosphate-impermeable volume of normal staphylococci in 0·1 M-NaCl was found to be 2·42 ± 0·05 ml./g. cell dry wt., in tolerably good agreement with the figure 2·67 ± 0·01 ml./g. for the close-packed cell volume less 26%. After treatment with dilute trichloroacetic acid or *n*-butanol, the phosphate-impermeable volume decreased to 0·76 ± 0·07 ml./g. cell dry wt., which, on correction for loss of diffusible constituents, gave 0·93 ± 0·09 ml./g. (Mitchell, 1953*a*). These observations were in accord with the view, based upon chemical analyses of the components of the cell envelope, that the osmotic barrier of staphylococci is a lipoprotein layer *c.* 15 mμ thick which is supported by an external cell wall *c.* 25 mμ thick (Mitchell & Moyle, 1951). There is therefore little doubt that an osmotic barrier impermeable to phosphate ions exists near the surface of the cells. This would imply either that the resting exchange of phosphate must occur by exchange-diffusion across the barrier, or that the so-called inorganic phosphate must be adsorbed outside the barrier. We can calculate that the effective internal cell volume was 1·66 ± 0·07 ml./g. cell dry wt. and that the normal inorganic phosphate content (*c.* 150 μmole/g.) would give a concentration of *c.* 0·1 M-phosphate in the internal medium. The concentration of phosphate that would be required if it were all present outside the barrier seems almost to preclude this possibility. However, a more direct confirmation of the occurrence of exchange diffusion was sought by studying the sensitivity of the exchange reaction to inhibitors.

If the exchange-adsorption hypothesis were correct, the so-called inorganic phosphate of the cell would be held by combining groups corresponding in number to the acid-soluble inorganic phosphate molecules. The inhibition of the exchange of phosphate on a combining group would not be expected to occur unless the inhibitor molecule were close to the combining group. Hence, unless action at a distance were postulated, the number of molecules of inhibitor required to retard the exchange reaction should be of the same order as the number of molecules of acid-soluble inorganic phosphate. If, on the other hand, the exchange-diffusion hypothesis were correct, the exchange would be mediated by a relatively small number of carrier groups in the osmotic barrier. In this case, an inhibitor might retard the exchange reaction when the number of molecules present was similar to the number of carrier groups and therefore small in comparison to the number of molecules of acid-soluble inorganic phosphate. Out of a large number of inhibitors, the heavy metals and their derivatives were found to be the most potent group, headed by phenyl-Hg^+. The relationship between the degree of inhibition of the exchange reaction and the concentration of phenyl-Hg^+ was represented by $K=Mn/(100-n)$, M being the amount of phenyl-Hg^+, n the percentage depression of the rate of phosphate exchange and K a constant. This indicated a reaction of the type $M+X=MX$, X representing the sites which, when combined with inhibitor (as MX), cause inactivation of a corresponding proportion of the units controlling the exchange of phosphate. The value of K was 2·2 μmole/g., and since the cells contained 147 μmole acid-soluble inorganic phosphate per g., the value of X per 100 molecules of acid-soluble phosphate or hypothetical adsorption sites was only 3 (Mitchell, 1953*a*).

There remained little doubt that the exchange of phosphate across the osmotic barrier in resting cells is due to some kind of exchange-diffusion system, and that the uptake of phosphate during respiration is due to a coupling of the exchange-diffusion system with other reactions. A more detailed study of the exchange system in resting cells was therefore pursued (Mitchell, 1953*b*).

The effectiveness of inhibitors upon the exchange reaction was studied to determine the specificity of the reaction for phosphate and to obtain information about the mechanism of inhibition which might be relevant to the mechanism of transport of the phosphate molecules across the osmotic barrier. Out of a total of some forty anions which were tested, some such as chlorate and borate acted as inhibitors, but only arsenate was able to substitute effectively for phosphate in the exchange reaction. A remarkable feature of the behaviour of arsenate, however, was that although it entered into the exchange system somewhat less readily than phosphate, it did so with the same efficiency as phosphate: In other words, the same one-to-one exchange is observed between arsenate and phosphate as between phosphate and phosphate. It is therefore possible to cause active transport of arsenate in exchange for phosphate. By washing the cells in 1 mM-arsenate, the internal inorganic phosphate, initially at a concentration of *c.* 100 mM, was found to pass out in exchange for arsenate which, towards the end of the exchange process, moved from an external concentration of 1 mM to an internal concentration approaching 100 mM. The exchange of arsenate and phosphate in this system is analogous to the cation exchange for which Ussing has postulated exchange-diffusion; but it should be appreciated that the tightness of the coupling of the arsenate-phosphate exchange is of a much higher order than that reported (Ussing, 1949) for the cation exchange.

Of the inhibitory anions, 2:4-dinitrophenate was one of the most interesting. At a concentration of 1 mM at pH 7 it caused *c.* 50% depression of the rate of phosphate exchange, approximately the same as the depression of endergonic processes dependent upon glucose fermentation (Gale, 1951). Aureomycinate inhibited phosphate turn-over to about the same extent as 2:4-dinitrophenate. It seems possible that the exchange reaction upon which 2:4-dinitrophenate and aureomycinate act in the *Staphylococcus* may have its counterpart in the oxidative phosphorylation systems of tissue particles which are so effectively decoupled by these reagents (Loomis & Lipmann, 1948; Loomis, 1950).

Following up the inhibitory action of the heavy metal cations, it was found that inhibition was caused by substances such as iodine or bromine at pH 5·5, or by chloroacetophenone or *N*-ethylmaleimide at pH 7, but not by iodoacetate—indicating that the exchange-diffusion reaction involves thiol groups of low reactivity. The inhibition of the exchange reaction by the decoupling agents and by the reagents reacting with thiols suggested that, although the exchange of phosphate across the osmotic barrier did not in itself involve an over-all free-energy change, a small residual metabolism might be necessary to allow the exchange reaction to occur, as has been suggested for the cation exchange in tissue particles (Davies, 1954). It was found, however, that under conditions in which residual metabolism was reduced to a level where it could not be detected manometrically, the exchange of phosphate continued at the normal rate. The rate of exchange was therefore presumed to be determined by thermal movements such as those considered by Ussing (1949) to operate the carrier groups in the exchange-diffusion systems, and not by the rate of triggering by some associated energy-consuming metabolic reaction. This being the case, it was thought that kinetic studies might be amenable of interpretation (Mitchell, 1953*b*).

At an external phosphate concentration of 1 mM, the velocity of the exchange reaction exhibited a sharp maximum at pH 7. This maximum, however, was a function of the external phosphate concentration, the optimum rate increasing and the optimum pH moving towards 9 as the external phosphate concentration was increased towards 100 mM. The dependence of the rate of exchange upon the external phosphate concentration at constant pH and ionic strength followed closely the classical Michaelis & Menten (1913) law, giving a value of 1·6 mM for the Michaelis constant (K_m) at pH 6·8 and ionic strength 0·1. When expressed in terms of total phosphate concentration the K_m value was a function of pH, but when expressed in terms of the concentration of $H_2PO_4^-$ ion, it was estimated to have the value 0·8 ± 0·1 mM independent of pH over a range from pH 5·5 to 8·5.

It seems probable, therefore, that the $H_2PO_4^-$ ion and not the $HPO_4^=$ ion enters the exchange reaction. The rate of the exchange reaction appears to be a linear function of the degree of saturation of an externally accessible reactant with $H_2PO_4^-$ ions in exactly the same way as the rate of an enzyme reaction is a linear function of the degree of saturation of the enzyme with its substrate. This does not necessarily mean that the externally accessible reactant is an enzyme, for in the exchange-diffusion system of Ussing, provided the movement of the carrier compound from one side of the membrane to the other were the rate-limiting step, exactly the same type of kinetics would be expected. However, the phosphate-exchange system differs from Ussing's in a most important respect. It has been pointed out by Ussing (1949) that when the carrier compound of his exchange-diffusion system is not saturated with its substrate on one side of the membrane or the other, considerable leakage of the substrate across the membrane should occur. According to Ussing's exchange-diffusion model, the K_m (0·8 mM) of the phosphate-exchange reaction would represent the dissociation constant of the carrier for the $H_2PO_4^-$ ion; yet, even when the external $H_2PO_4^-$ concentration was lowered to 0·1 mM a strictly reciprocal exchange of phosphate still occurred. The phosphate-exchange system must therefore differ from Ussing's model in a way which makes it more strictly coupled. This might be accomplished by a number of obvious mechanisms. Probably the simplest and most attractive hypothesis, however, is as follows: Phosphorylated carriers are present in the osmotic barrier the phosphate groups of which, due to thermal movements of the carriers, come into contact with the media on either side where an enzyme-catalysed exchange may occur between the phosphate groups of the carriers and phosphate ions. The essential difference between Ussing's system and this one is that in the latter the free energy of formation of the carrier-ion complex (or compound) is assumed to be conserved during the enzyme-catalysed exchange of the ion, the carrier-ion complex thus not being in equilibrium with its dissociation (or hydrolysis) products on either side of the barrier as is the case in Ussing's system.

It was found that the value of K_m (expressed as a concentration of $H_2PO_4^-$) was constant to within ± 10% over a temperature range from

5 to 25° C. Thus, the heat of formation of the external phosphate complex must be small. On the other hand, the temperature coefficient of the rate of the reaction at constant external phosphate concentration was greater than 10, and gave a constant Arrhenius energy of 38,000 cal./mole over the temperature range from 5 to 20° C. The heat of activation of the exchange process (equal to the Arrhenius energy—RT) is therefore a little over 37,000 cal./mole. This heat of activation represents the increase in total heat required for the thermal movements of the carriers which effect the translocation of the phosphate groups across the osmotic barrier in the model system proposed above.

It is hardly necessary to point out that the value of the heat of activation of the exchange reaction is much higher than that usually observed in diffusion processes. In fact, it is unusual for reactions with such high heats of activation to proceed at a measurable rate at room temperature. This, however, is equivalent to the statement that the free energy of activation of the exchange reaction (upon which the absolute reaction rate depends) is probably much smaller than the heat of activation, the larger part of the heat of activation being due to an entropy change as in reactions such as the denaturation of proteins (Glasstone, Laidler & Eyring, 1941). The thermodynamic data are thus in accord with the view that the osmotic barrier, across which the carrier effects the translocation of phosphate and of which the carrier forms an integral part, is a well-organized internally bonded structure. The translocation reaction may be regarded as a kind of reversible denaturation of the osmotic barrier.

We have no definite information at present as to the chemical nature of the substance which carries the phosphate across the osmotic barrier of the *Staphylococcus*. Gourley (1952) has suggested that adenosine triphosphate (ATP) may be the intermediate which carries phosphate across the membrane of red blood corpuscles; for, during active uptake of isotopic inorganic phosphate the isotope enters the internal ATP fraction faster than the internal inorganic phosphate fraction. The phosphate-exchange system proposed for the *Staphylococcus* might operate with ATP as the carrier and phosphokinases as the enzymes, although efforts to locate an externally situated phosphokinase have failed. If this were the case, however, the strongly lipophobic properties of ATP would almost certainly have to be masked by some large molecule such as a protein of low water solubility, in company with which the ATP would have to pass across the osmotic barrier by a translational or rotational movement of the large molecule. Although such a system is not more complicated than we might anticipate for the organization of biological transfer reactions, it includes rather more speculation than the present experimental evidence will safely bear. The fact must not be overlooked that Gourley's results might be explained adequately if the intermediate of the carrier system were to pass its phosphate direct to adenosine diphosphate inside the cells during active phosphate uptake.

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ANION RESPIRATION

THE EXPERIMENTAL BASIS OF A THEORY OF ABSORPTION, TRANSPORT AND EXUDATION OF ELECTROLYTES BY LIVING CELLS AND TISSUES

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I. INTRODUCTION

The processes of absorption, accumulation and transport of electrolytes are of universal importance in all living organisms. They appear more prominently in plants because (1) plant cells accumulate large quantities of salts from dilute media, and (2) the long-line transport of salts, known as sap movement, is closely related to this cellular power of active salt transport. 'Active' here means requiring supply of energy, active transport thus covering both the case of accumulation against a concentration gradient and also polar movement of ions.

The question as to the possible mechanism of active transport of cations and anions has aroused much speculation. The problem may be looked upon from different angles, and it is probable that active transport of ions may be realized in different ways. At present, only the theory of anion respiration is supported by experimental material of a magnitude which permits the building up of a serviceable theory. My laboratory has devoted more than twenty years of extensive experimental work to lay the ground for the theory of anion respiration, and since 1941 brilliant contributions have emanated from the laboratory of R. N. Robertson. Important studies were also made in the laboratory of D. R. Hoagland. Owing to limited space I will restrict myself to a survey of the main facts and conclusions.

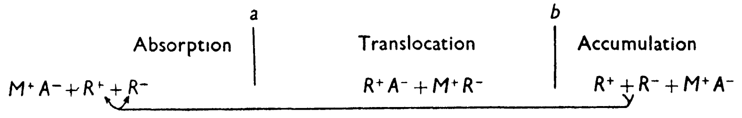
Roots of cereals, primarily spring wheat, have served as the experimental material in my own laboratory. Roots are very suitable objects, because they act as the salt pumps of the whole plant and thus reveal both sides of the problem, i.e. both absorption and exudation of ions. In some other laboratories, e.g. that of Robertson, slices of storage tissue (carrots, etc.) served as the main experimental material. It is of considerable interest to note that the chief results from such different tissues agree fairly well.

II. THE PRINCIPLE OF ION CARRIERS

Several earlier workers (e.g. Osterhout, Hoagland) observed that the cations and anions of a salt were frequently absorbed in non-equivalent quantities. It is a well-known fact that much more nitrate than calcium is absorbed from calcium nitrate, whereas from potassium nitrate the quantities of absorbed cations and anions are more equal. It has been shown that the balance may be moved in one direction or the other by pre-treating the objects with salts; roots charged with calcium absorb very little of this cation, whereas the absorption of nitrate is only little influenced (Lundegårdh, Burström & Rennerfelt, 1932; Lundegårdh, 1937). These observations support the idea of a separate entrance of the cations and the anions into the cell.

Outgrown plant cells have a large sap space (vacuole) surrounded by a thin layer of protoplasm, the peripheral part of which is the protoplasmic membrane. This construction of the cell permits a sharp definition of active accumulation; it is the absorption of $c_0M^+A^-$ from the medium and its accumulation as $c_1M^+A^-$ in the cell sap, c_1 being larger than c_0 and M^+A^- symbolizing a neutral salt. The energy (E) required for the necessary osmotic work is expressed by the formula $E = RT \ln c_1/c_0$. For a gradient $c_1/c_0 = 100$, not uncommon in roots, E amounts to *c.* 2700 cal./mol. at 20° C.

Most workers in the field of salt accumulation accept the idea of *ion carriers*, or large organic ions in the protoplasm, symbolized as R^+ and R^-, endowed with more or less specific attraction to salt ions. The idea of ion carriers goes back to Osterhout and other earlier investigators. It has been further developed by the work in my laboratory. The entrance of salt ions into the cell is figured as the formation of compounds M^+R^- and R^+A^-. The accumulation of the free salt M^+A^- is figured as the reversal process, implying the dissociation of the carrier compounds somewhere inside the protoplasmic membrane. The complete process of salt uptake follows the scheme:



(1)

The supply of accumulation energy may be at *a* and/or *b*. It is, furthermore, clear that only *one* of the ions of the salt *MA* requires a supply of accumulation energy. Provided anions are actively accumulated the acid H*A* will spontaneously decompose the compound *MR*, resulting in free salt *MA* and regenerated carrier H*R* able to combine with a new cation from the medium. It is not even necessary that the carriers R^+ and R^- are rotating as indicated in the above scheme, because repeated ion exchange (see below) will serve the same purpose.

The outlined mechanism very probably exists in roots. Some of the investigations performed in my laboratory have been devoted to the elucidation of the question of cation carriers R^- and the spontaneous reaction $R^- + M^+ \rightarrow MR$.

In the first decade of this century, when I started work on salt uptake in plant roots, much attention was paid to the interaction of salts and protoplasm along the lines of 'salt permeability', implying the passive diffusion of salts into the living cells. These studies and speculations followed upon early work in colloidal chemistry, later followed by the modern concept of macromolecules and their physical chemistry. The protoplasmic membrane is to-day pictured as a more or less flexible network of large molecules, forming a mosaic pattern and composed of a variety of chemical compounds. Some of these are certainly non-dissociated, others are more or less ionized, forming electrically charged islands in the electro-neutral ground substance of the protoplasmic membrane. In the surface of the plant roots the ratio of R^- to R^+ groups appears to be 100 or more. Varying conditions of dissociation may, however, severely change the balance.

In an aqueous medium the carriers are balanced by small movable ions of water or neutral salts. As shown below, some of the carriers, if not all, are more or less specific to the attracted small ions of opposite charge, thus inducing a selective absorption. The bonds between carrier and movable ion are similar to those characteristic of 'adsorption' and obey the law of mass action (see Lundegårdh, 1941). The phenomenon of ion exchange is a manifestation of these principles.

Ion exchange is the main path of non-metabolic salt absorption by protoplasm as was demonstrated in extensive experiments (see below). Simple diffusion of salts plays a more subordinate role, as is shown by the absence of diffusion potentials (Lundegårdh, 1938) and by direct experiments with strong solutions with respiration inhibited (Hoagland & Broyer, 1942). Judging from experiments on the exchange of cations the total 'exchange capacity' (sometimes called 'free space') is of such a magnitude that it probably includes most of the protoplasm. Hope & Robertson (1953) claim that the concentration of the R^- groups may be as high as 10^{-2} mol. Measurements of the surface potential give values of 10^{-2}–10^{-3} mol. as shown in my work on these questions.

III. THE SURFACE POTENTIAL OF THE PROTOPLASM

The dissociation of the protoplasm creates an electrokinetic potential difference between the surface layer and the medium. In the case of single cells, e.g. bacteria or erythrocytes, it can be measured electrophoretically. In the case of whole organs, such as roots, it may be measured as electromotive power. In my experiments during 1938–41 the root potential was measured by means of a cathode-ray oscillograph, permitting the recording of very rapid changes. The surface potential of root hairs can be measured electrophoretically (Lundegårdh, 1941) if they are observed by microscopy in a field of alternate current (2 V. and 20 cycles/sec.). The amplitude of the oscillations in the field is a measure of the magnitude of the charge. It was shown that the amplitude is approximately proportional to the pH, if diluted HCl is used as medium, the vibrations ceasing at pH 3 in the case of wheat roots (see Lundegårdh, 1941, fig. 4). Whole roots submerged in a dilute nutrient solution to a depth of 20–30 mm. show a negative electrokinetic potential of *c.* 60 mV. That the site of the charge is the surface of the living roots was demonstrated from the extremely rapid time course of potential changes induced by a change of the ionic composition of the medium.

By observation of the decrease in potential on increasing the H-ion concentration of the medium it is possible to plot a titration curve of the protoplasmic membrane and from this to calculate its apparent dissociation constant (see Lundegårdh, 1941, fig. 12). The surface of the living epidermis cells of the wheat roots behaves as a comparatively strong acid, the pK being 1–2. No carboxylic acids attain this high dissociation, only substituted phosphoric or sulphonic acids. The latter are absent from the cell surface. It was accordingly concluded that phosphate groups in organic linkage are probably serving as carriers of cations in the root surface. This conclusion is to some extent supported by a later discovery of nucleotides among the normal exudates from the living root surface (Lundegårdh & Stenlid, 1944). Also observations on bacteria support the idea of nucleotides present in the surface of the cell.

According to our conception of the mosaic pattern of the surface of the protoplasm the electrokinetic potential is an expression of the dominating acid dissociation, and it behaves essentially as a Donnan potential. If the medium is changed from distilled water or a very diluted mineral acid to solutions of neutral salts, the negative potential is lowered towards zero at increasing concentration of the salt and finally changes to positive. This fact is explained as follows. In the absence of metallic cations in the medium the R^- groups are balanced by H^+. The root surface behaves as an H-ion electrode, the measured potential being an expression of log H_i^+/H_0^+, if H_0^+ represents the cH of the medium and H_i^+ the cH of the root surface. If a neutral salt is added the *M*-ions exchange with H_i^+ to an extent determined by the activity and a factor *a*, representing the specific adsorption qualities of the cation in question (see Lundegårdh, 1941). The potential is accordingly lowered, the drop being approximately proportional to the logarithm of the salt concentration (see Lundegårdh, 1940, fig. 5; 1941, fig. 15).

During prolonged exposure to a salt solution these exchange processes in the surface, which are so excellently reflected in the electrokinetic potentials, are followed by similar processes in the bulk of the protoplasm resulting in a chemically measurable cation exchange between a root and the surrounding medium (see Lundegårdh *et al.* 1932; Lundegårdh, 1945, 1950*a*). These observations teach us that cation carriers are also present in considerable quantities in the bulk of the protoplasm. A useful tool for measuring the quantities of cations absorbed by the root and the quantities of other cations simultaneously given off is an application of the method of quantitative spectrum analysis which I introduced (1929). By means of this technique it was shown (Lundegårdh *et al.* 1932) that in the alkali series K is most intensively absorbed, followed by Cs and Rb. Of the highly hydrated Li less is absorbed, and of Na, the hydration of which is much higher than that of K, only small quantities are absorbed. The simultaneously exuded quantities of cations reflect the power of ion exchange.

Charging of the roots with Ca considerably increases the exchangeable quantities of this ion. As expected, H^+-ions have a high exchange power. From electrically recorded salt absorption experiments (Lundegårdh, 1949*c*) it was calculated that a solution of 0·0005 mol. HCl exchanges quantities of K from the roots which considerably exceed the quantities of this ion actively absorbed from a 0·001 mol. solution of KCl. The predominating power of cation exchange is reminiscent of soil colloids, with the difference that living protoplasm absorbs cations more intensively than soil colloids do. The dominating attraction of cations, however, does not exclude a certain exchange of anions, as may be observed from potential experiments with salts of different anions and the same cation, or from the reversal of charge in strong salt solutions. The normally low surface concentration of the anion carriers R^+ (see above) hampers the analytical determination of the anion exchange, but there is little doubt that also in respect of anions exchange processes are at work. In several papers I have pictured the exudation of salts from the protoplasm into the vacuoles as a passive exchange process. Some investigators, e.g. Hoagland, Arisz (1952) and others, believe in an active excretion through the tonoplast.

Their arguments are not convincing, and a fact directly speaking against an active excretion into the vacuoles is the rapid outflow from the tissue at times of salt starvation (see Lundegårdh, 1945, fig. 2). The observed facts support the idea of an exchange equilibrium between protoplasm and vacuole, the latter serving as a transitory store of salts. There is no conflict between this idea and the frequently observed slow rate of diffusion through the tonoplast.

IV. NON-METABOLIC ABSORPTION AND EXUDATION OF IONS. POSSIBLE MECHANISMS OF SALT ACCUMULATION OTHER THAN ANION RESPIRATION

Rotation of the carrier molecules at the cell surface serving as ion acceptors will bring the attached ions into the protoplasm, and they are then taken over by other carriers circulating in the protoplasm (fig. 1). One may speak of 'adsorption tracks' or 'surface gliding of ions' if the ions are intermittently pushed and pulled along structures (see the scheme in Fig. 2). It was experimentally shown (Lundegårdh, 1945, 1950) that this movement is non-metabolic, i.e. not linked to respiration. In the case of roots, metallic

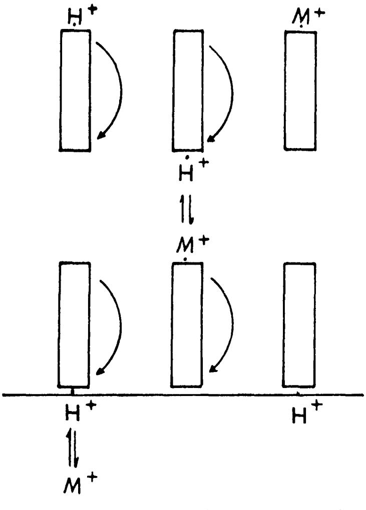


Fig. 1. Scheme of the absorption of cations by carrier molecules and their transport inwards by means of rotation of the carriers.

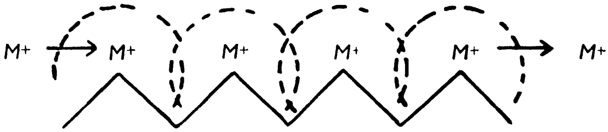


Fig. 2. Scheme of rapid transport of ions along 'adsorption tracks'.

cations will dominate over nutrient anions owing to the predominating acidic qualities of the cell surface, but certain quantities of salts will nevertheless always be available for passive transport by means of the transpiration stream or for metabolic purposes.

In the case of metabolic taking over of ions from their carriers to other chemical systems, one may speak of active salt absorption at *b* in scheme (1). But the common metabolic activity of the cell offers more direct mechanisms of accumulation. Two of these may be mentioned here, namely, variations in the dissociation of the carriers *R*, and variations in the acid/base balance.

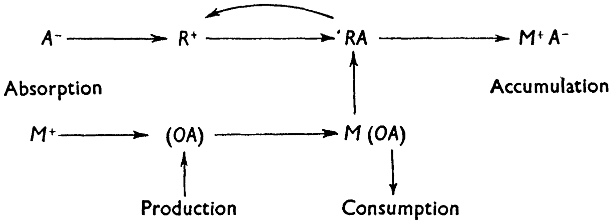
The quantitity of carried ions (MR and RA) corresponds to the quantity of H^+ and OH^- held in exchangeable state by the large carrier ions. The dissociation is regulated by the pH of the surroundings, and a lower pH at the cell surface in combination with a higher pH in the cell sap, as realized in many plant cells, undoubtedly favours an import of metallic cations. But such conditions favour either cations *or* anions, never both. If, however, the dissociation constant (k) of the carrier is changed, conditions may be fulfilled for a real accumulation mechanism, according to the following scheme ($k_1 > k_0$):

$$\begin{tikzcd}
 & R^-_{k_1} \arrow[d] & & & \\
M^+ \arrow[r, "\text{Absorption}"] & {} \arrow[r] & MR_{k_1} \arrow[r, "\text{Metabolic}", "\text{Transformation}"'] & MR_{k_0} \arrow[r, "\text{Accumulation}"] & M^+
\end{tikzcd} \qquad (2)$$

Transformation

A change in the acid dissociation constant of R^- groups is probably induced by certain ions, e.g. Ca, Mn and BO_3, because these ions maintain a high negative potential of the root surface whereas K-ions act in the reversed direction (Lundegårdh, 1940). An increase of the concentration of R^- in the surface will, however, give the same result. Changes in the dissociation constant of large molecules, induced by small stoichiometric changes, are well known. In the chain of reactions participating in aerobic respiration, changes of the dissociation constant occur at many points, especially in connexion with the phosphate metabolism.

Variations in the acid/base balance have been studied from many angles. Of special interest are metabolic regulations of the internal pH of root cells (Ulrich, 1941; Burström, 1942). At storage of organic acids, e.g. malic acid, in the cells, metallic cations are released from the protoplasm in exchange for H-ions. More cations are concomitantly absorbed from the medium. If some of the acid is consumed in the tricarboxylic cycle the remaining metallic cations will release adsorbed anions from the protoplasm and more anions will then be absorbed from the medium. As freely movable anions are not so easily available, owing to the dominating acidoids in the protoplasm (see above), supernumerary metallic cations may also be exuded from the root surface. For maintaining a continuous accumulation of salts a metabolic mechanism must be thought to produce acids in one region or layer of the cell and to consume acids at another place according to the following scheme (*OA* = organic acid):



(3)

Some authors believe that such a scheme is realized in connexion with the activity of the cytochrome system (Helder, 1952; Vervelde, 1952), but this is a mere speculation. In cells deprived of a cytochrome system *organized for polar transport* (see below) mechanisms operating by means of potential gradients controlled by dissociation and/or continuously working cycles of the acid/base balance may possibly exist. But such cases will have to be experimentally demonstrated. A specific absorption of anions may be expected if they are caught by a special carrier also serving as a mechanism of accumulation. It is commonly believed, though never strictly proved, that phosphate is absorbed by the aid of phosphorylation. Phosphorylation at the surface of the cell and dephosphorylation inside of it will of course serve as a suitable mechanism for accummulation of phosphate. The fact that the absorption of sugar is probably linked to a special mechanism working in the cell surface (cf. Lundegårdh & Burström, 1944) supports this idea, because a reversible phosphorylation is probably acting here too.

From the point of view of *specific ion carriers* attention is called to the coenzymatic function of a number of cations, Ca, K, Mn and Mg. The selective absorption of cations has been known for a long time. In experiments on the absorption of the members of the alkali series (Lundegårdh *et al.* 1932) it was observed that K was absorbed more rapidly than would be expected from its physico-chemical properties (hydration, dimensions, etc.). From equimolar solutions (0·0025 m.) of chlorides the relation K/Na amounted to 24. Li is considerably more hydrated than Na, but the relation K/Li nevertheless amounted to only 1·8. The slow entrance of Na is obviously caused by a low percentage of carriers suitable for this ion. The selectivity is frequently a group character. It was observed that K and Rb behave very similarly (Burström, 1937). The recent results of Epstein (1952) only corroborate the earlier research work.

V. THE SAP MOVEMENT

Salts which have penetrated the epidermis layer are comparatively rapidly distributed through the root tissue. This internal transport is partly independent of respiration and glycolysis (Lundegårdh, 1950). It is probably facilitated by the absence of potential differences between the internal surfaces of the cells and by exchange processes. The rapidity of ion exchange processes was experimentally demonstrated (see above). Owing to the mobility of salts stored in the root cortex these are continuously emptied into the open central vessel of the roots (Fig. 3). Under anaerobiosis the exudation continues, but with decreasing speed and with a certain preference for cations (Lundegårdh, 1945, 1950).

The non-metabolic character of these internal movements of salts is emphasized by the low temperature coefficient, the Q_{10} amounting to only 1·5–1·6, whereas the Q_{10} of active accumulation amounts to 2·25–2·37 (Lundegårdh, 1950, p. 146). Wanner (1948) found a Q_{10} of 1·4 for the absorption of K-ions from the medium, but $Q_{10} = 2$ to 2·5 for the absorption of the nitrate ion. These results point to the existence of separate mechanisms for the accumulation of cations and anions through the epidermis, but the translocation inside the epidermis may be in part non-metabolic as shown by a lower Q_{10} for *both* ions. My own results show that salts may be passively transported through the root tissue by means of non-metabolic processes (probably a diffusion enhanced by ion exchange along 'adsorption tracks'). Only active transport yielding accumulation against the concentration gradients requires the assistance of metabolic processes.

The concentration of the exuded sap is normally higher than that of the nutrient solution (Lundegårdh, 1943, 1945, 1950*b*). In wheat it usually amounts to 15–25 mmol. KNO_3 (the dominating salt) per litre, but may rise to 37 mmol. $\times$ l.$^{-1}$. This concentration is, however, lower than the mean concentration of KNO_3 in the root cells, or 40–80 mmol. $\times$ l.$^{-1}$. From the point of view of exudation into the vessels the process consequently does not involve osmotic work. As shown in a discussion of the kinetics of salt exudation (Lundegårdh, 1950, p. 104), even the exudation of much higher concentrations need not necessarily involve active work. This conclusion may hold good also for the highest exudation value ever observed in my experiments, viz. *c.* 120 mmol. $\times$ l.$^{-1}$ from roots treated with 0·01 mol. NaF at pH 4·4. An indispensable condition for all this non-metabolic translocation of salts is of course that a certain level of concentration has been created by preceding *active* work (see below).

In principle there is no large difference between the exudation of salts from the vascular epithelium into the vessels and the exudation of K from the epidermis into a diluted solution of HCl, mentioned above. Also quantitatively the two cases are very similar. The influx and outflow of cations from the epidermis is essentially an ion-exchange process, due to existing gradients of the concentration (activity) of the involved ions. The same scheme may also be applied to the internal exudation from the vascular epithelium. The fact that the outflow from the epidermis is mainly restricted to cations, whereas normally neutral salts are exuded from the vascular epithelium, points to a less acid reaction of the exuding surface of the latter, favouring a more equivalent activity of cations and anions according to the Donnan principle. The outflow of salts may be pictured as a 'canalized leakage' (see Lundegårdh, 1950*b*, p. 112), viz. a permeability restricted to salt ions, escaping from the ionized points of the cell surface.

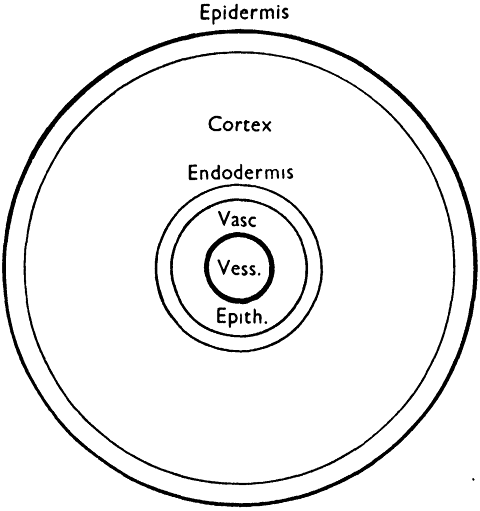


Fig. 3. Diagram of the main tissue layers in a grass root. From the surface to the centre: (1) epidermis (single layer of cells without intercellulars); (2) cortex (several layers of large cells with large intercellulars); (3) endodermis (single layer of cells without intercellulars); (4) vascular epithelium (tissue of narrow cells surrounding the vessels); (5) central large vessel.

Organic substances are not exuded by the vascular epithelium of wheat seedlings. The outside of the epidermis behaves differently also in respect of exudation of organic substances which are regularly exuded in varying quantities (Lundegårdh, 1932; Lundegårdh & Stenlid, 1944; Stenlid, 1950). The fall in the concentration in the tissue caused by the *internal* exudation into the vessels may be followed by intermittent analysis. It is reflected in a falling concentration in the exudate. Because water is exuded into the vessels not only owing to the osmotic conditions of salt exudation but also owing to metabolic processes, e.g. the glycolytical disappearance of sugar (see Lundegårdh, 1949*b*, 1950*b*), interesting variations of the concentration of the sap may be induced under the influence of inhibitors affecting these processes, but these questions are outside the scope of the present survey.

VI. DEFINITION OF THE ANION RESPIRATION

Cations and anions may be absorbed, transported and exuded on the basis of what we have called non-metabolic processes, but a *continuous* exudation of salts in the ascending sap requires a continuous renewal of the level of concentration in the exuding cells, *a condition not realized with inhibited respiration.* Under anaerobic conditions salts exude into the vessels for several hours, but to a slowly sinking degree, and no salts are absorbed through the epidermis to restore the sinking osmotic gradient. Only an active anion respiration is capable of filling the salt magazines of the root cells.

The discovery of quantitative relations between the active absorption of salts and respiration was published in 1933 by Lundegårdh & Burström. Independently, Steward (1932) had shown that aerobic respiration (O_2 consumption) is a necessary condition for salt accumulation in slices of storage tissue. Later Steward, together with Hoagland and other investigators, insisted upon the thesis that there are no quantitative relations between aerobic respiration and salt accumulation. Lundegårdh & Burström (1935) and Lundegårdh (1937 and later papers) were able to distinguish two groups of aerobic respiration, the *ground respiration* and the *anion respiration*. The former is not inhibited by 0·001 mol. cyanide and shows no direct relation to salt accumulation. The latter is inhibited by very low concentrations of cyanide, as also by NaN_3 and CO, and its intensity is closely related to the intensity of salt absorption.

The quantitative relation between absorbed salt and the cyanide-sensitive respiration is primarily an effect of the anions. Already the pioneer experiments showed a clear proportionality between absorbed anions and one fraction of the respiration. No clear proportionality was traced in respect of the simultaneously absorbed cations. In later investigations (Lundegårdh, 1937) with roots pretreated with Ca in parallel experiments with $Ca(NO_3)_2$ and KNO_3 in equinormal concentrations found approximately the same absorption of nitrate and the same anion respiration, whereas no cations were absorbed from the $Ca(NO_3)_2$ but quantities of K from the KNO_3. Other experiments with bicarbonates of K, Ca and Ba showed no anion respiration, but a considerable uptake of cations. It is important to note that all these experiments were performed with carefully treated 2–3 weeks old intact seedlings, 'desalted' by pretreatment with distilled water under illumination in photothermostats. The experimental vessels were specially constructed to maintain perfect aeration of the intact plants ('circulation vessels'). Each experiment lasted from 10 to 20 hr. In later experiments also detached root systems were submerged in the solution and shorter experimental times were used, usually 1 hr. The absorption and exudation of cations and anions was closely followed by spectrographic and chemical analysis of solutions and in certain cases also of the roots. In some later experiments the electrical conductivity was also used as a measure of the concentration of the medium. It is important, however, to check the conductivity measurements by chemical analysis (Lundegårdh, 1949*c*).

VII. THE ANION RESPIRATION AS AN ION CATALYSIS

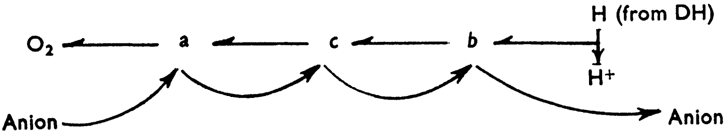
The idea of a respiratory system, operating with active Fe as the source of accumulation energy, was developed at an early date (Lundegårdh & Burström, 1935; Lundegårdh, 1935, 1937). It was later shown that another heavy metal, Mn, had a considerable influence on the ground respiration and on nitrate assimilation (Lundegårdh, 1939; Burström, 1939). The iron catalysis was later assumed to be identical with the cytochrome-cytochrome oxidase system. This idea was accepted and further developed by R. N. Robertson (1941 and later papers). Robertson devoted extensive attention to salt respiration in slices of storage tissue, primarily of carrot. He was able to demonstrate the existence of an anion (or salt) respiration and a ground respiration, both quite similar to the corresponding processes in roots. Robertson observed the initial non-metabolic phase of salt absorption, the rise and the fall of the anion respiration with the salt content of the medium, its high sensitivity to cyanide, and the restricted passive leakage of anions from the cells.

The theory of anion respiration in its present shape was outlined by introducing the hypothesis that the active transport of anions is causally linked to the transference of electrons in the cytochrome system (Lundegårdh, 1945). The leading idea is the postulate that variations in the charge of two reacting cytochromes, Ct_1 and Ct_2, induced by the transference of one electron according to the formula

$$Ct_1.Fe^{2+} \rightleftharpoons Ct_2.Fe^{3+} + e, \tag{4}$$

are reflected in the distribution of the movable anions in the surroundings, one anion being attracted by $Ct_2.\mathrm{Fe}^{3+}$ at the same moment it is uncoupled from $Ct_1.\mathrm{Fe}^{2+}$.

In an intact cytochrome-cytochrome oxidase system the electrons are moved from a dehydrogenase system (DH; as a rule succinic dehydrogenase) through the potential ladder of the cytochromes to oxygen. As shown in the following scheme:



(5)

anions will concomitantly be moved in the opposite direction. The final acceptor of the electrons is the O_2. The final acceptor of the anions are cations circulating in the surroundings of *b*. Protons are produced when the hydrogen of the reduced dehydrogenase is oxidized by cytochrome *b* (or some intermediate factor; see below and Lundegårdh, 1952, 1953).

If the cytochrome system is part of a membrane structure (Lundegårdh, 1950), with the oxidase facing the medium and cytochrome *b* facing the place of accumulation, conditions will be fulfilled for an active absorption of salts from the medium into the cell.

VIII. THE QUOTIENT Q an./O_2

Robertson (1941) called attention to the fact that four electrons are required for the complete reduction of one molecule O_2 according to the formula

$$4H^+ + 4e + O_2 = 2H_2O. \qquad (6)$$

If one electron is exchanged for one monovalent anion the quotient an./O_2 or (mol. absorbed anions/mol. consumed oxygen) will at best attain the value 4. Robertson & Wilkins (1948) found in experiments with slices of carrot that in strong salt solutions Q an./O_2 rose to approximately 4 but never higher.

The fact that in roots Q an./O_2 seldom attains higher values than 1–2 (Lundegårdh, 1949) had now to be investigated. I had previously observed (Lundegårdh, 1937) that roots held in distilled water show a cyanide-sensitive fraction of the aerobic respiration amounting to some 50–75% of the anion respiration in salt solutions. This 'distilled-water respiration' (d.w.-respiration) was now more closely analysed (Lundegårdh, 1949*b*). It was shown that it decreases with increasing periods of washing of the roots in distilled water (see Table 1). A considerably more rapid decrease near to zero was rapidly obtained in 0·0005 mol. HCl, whereas a similar period in 0·005 mol. $KHCO_3$ raised the d.w.-respiration above the original value. The 'idling' of the anion respiration was explained as follows:

What is measured in a tissue is not only the metabolism of the surface layer in direct contact with the medium but primarily the bulk of the tissue, the single cells of which communicate only with surrounding cells.

Table 1. *Decrease of the cyanide-sensitive respiration by washing in distilled water or dilute acids*

Medium ...	Distilled water during				0·0005 M-HCl	0·005 M-$KHCO_3$
Time ...	1 day	2 days	4 days	5 days	1 day	1 day
Cyanide-sensitive respiration (relative)	100	56	36	27	11	131

The large quantities of salts accumulated in the single cells of the cortex have traversed a number of cell walls on their way from the medium. The solution in the cellulose walls and in that part of the protoplasm which is responsible for the exchange capacity is the medium from which the cytochrome system of the internal cells pumps its anions. Assuming a steady state

$$\frac{\text{Accumulated anions}}{\text{Anions} \times cCt} = K, \tag{7}$$

in which cCt is the effective capacity of accumulation of the cytochrome system, and K the 'coefficient of accumulation', we arrive at the conclusion that there is an intracellular solution of salts in a steady-state balance with the accumulated salts in the sap space of the single cells. This intracellular salt solution is of course a very small quantity, because even the total volume of protoplasm is very much lower than the total volume of vacuoles, but it will be able to furnish a rapid stream of water, as in transpiration, with certain amounts of salts (see Lundegårdh, 1945, 1950). Its main task, however, is to furnish the internal cells with material for salt accumulation.

Equation (7) teaches us that this movable non-accumulated fraction of anions exists also in roots held in distilled water and that cCt must be at work unless the level of accumulation sinks. Salts may under these circumstances be transported from regions of lower cCt value to regions of higher power of accumulation. It was shown experimentally that internal salt transport is accelerated by the anion respiration (see above on sap movement). The slowly decreasing d.w.-respiration reflects the sinking of the salt level in the tissue (Table 1).

In addition to inorganic salts, anions of organic acids participate in the d.w.-respiration. This is shown by the rapid decline of organic acids at low pH (HCl) and the corresponding rise in alkaline solutions ($KHCO_3$). An analysis of the content of malic acid in the tissue (unpublished results) corroborates this conclusion. The d.w.-respiration may consequently be interpreted as an anion respiration by means of 'native anions', representing internally transported salts plus organic acid, and to include also true 'idling', viz. the re-accumulation of anions passively leaking out from the cells (cf. Lundegårdh, 1937). After transference of the roots to a salt solution anions absorbed from the medium are added to the native anions thereby raising the total anion respiration to the top level which is attained if the supply of anions fully covers what is needed for a cytochrome system operating at maximal speed. It was calculated that absorbed anions participate with *c.* 25–30 %, circulating inorganic anions with about the same, and anions of organic acids with about 40–50 % of the total capacity of the accumulation mechanism in roots. A participation of organic acids (succinate) in the anion respiration is also assumed by Turner & Hanly (1949).

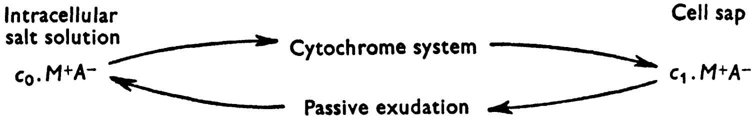
This analysis of the d.w.-respiration opens a possibility of explaining the differences of *Q* an./O_2 if different anions are compared. From equinormal solutions nitrate, chloride and sulphate were absorbed with sinking *Q*-values (Lundegårdh & Burström, 1933). Also the cations have an influence, easily movable cations as a rule raising, slowly movable cations lowering the *Q*-values (Lundegårdh, 1937). All these observations may be explained from a competition between absorbed anions and native anions. At low availability of absorbed anions, caused by low concentration or low ionic mobility, the native anions share a larger part of the total assembly of transported anions.

From the viewpoint of quantitative relations it is now important to know that *Q* an./O_2 maintains its value if the capacity of the cytochrome system is gradually slowed down by rising concentrations of cyanide, as shown in experimental series with nitrate and chloride (Lundegårdh, 1949*b*).

IX. THE SALT ACCUMULATION AS A STEADY STATE

In addition to equation (7) the salt situation of the root cells may be figured as a steady state between an uphill process, the anion respiration, and a number of downhill processes collected under the name of passive leakage or exudation (Lundegårdh, 1948). The passive exudation includes diffusion, ion exchange, reversed adsorption, destruction of carriers, etc.

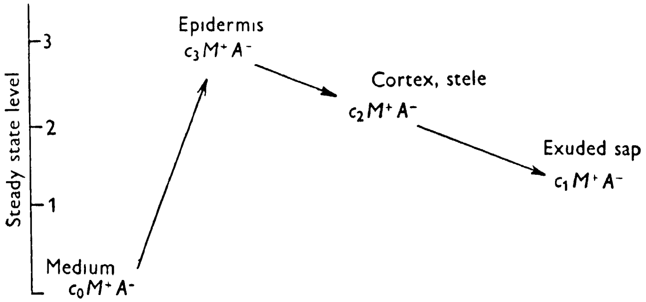
The steady state of single cells



(8)

The relation c_1/c_0 or the degree of accumulation is determined by the dimensions of the cytochrome system in the single cells and may accordingly be different in different layers of the root (see Fig. 6). Owing to its high osmotic pressure the epidermis is probably provided with a very active cytochrome system. The endodermis may possibly also develop a high level of accumulation, as a sluice of salts to the vascular epithelium. Very little is known, however, of these physiological-anatomical differentiations. The bulk of salts is certainly stored in the cortex. See the following scheme:

The steady state of the root tissue



(9)

X. SPECTROSCOPIC IDENTIFICATION OF THE CYTOCHROMES

Before 1950 the identification of the anion respiration mechanism with the cytochrome-cytochrome oxidase system was hypothetically built on the extremely high sensitivity of anion respiration and absorption to cyanide. Similar and more specific effects were found with other inhibitors of active iron, e.g. $\alpha\alpha$-dipyridyl (Stenlid, 1950). A good criterion of the presence of cytochrome oxidase is the inversion of the CO inhibition by light. Independent investigations with wheat roots (Sutter, 1950) and slices of carrot (Robertson & Wilkins, 1948) now showed a synchronous inhibition of the anion respiration and the absorption of chloride anions under the influence of a mixture of 95% CO and 5% O_2 and a similar synchronous recovery of both processes after illumination with strong white light.

Final evidence of the causal relation between the cytochrome system and the anion respiration mechanism was yielded in my recent studies of the absorption spectrum of the living roots (Lundegårdh, 1951*a*, *b*, 1952, 1953*a*–*c*). A special photoelectric spectrophotometer was constructed and built for this purpose, enabling the automatic recording of the absorption spectrum of a thick bundle (mostly 15 mm.) of roots under varying conditions (solutions of different oxygen pressure and salt content, of different inhibitors, at different temperature, etc.).

The cytochromes are fairly dominant in the absorption spectrum of the wheat roots. Of other coloured substances only carotenes show a prominent band at *c.* 482 mμ, whereas peroxidases (band at 404 mμ) and flavoproteins (band at *c.* 455 mμ) interfere only little in vigorously growing roots. It is important, however, to select such roots, because at certain periods of the year, e.g. October to February, the seeds frequently suffer from a reduced activity of the cytochrome system, combined with a reduced salt absorption. Under these circumstances other coloured substances, e.g. flavoproteins, are more prominent and the roots also contain quantities of non-specified haemin substances, possibly serving as precursors of cytochromes.

For identification of the cytochromes the oxidation-reduction spectrum, viz. the extinction of reduced cytochrome minus the extinction of oxidized cytochrome (see Lundegårdh, 1951, 1952), proved very useful. The difference spectra of the roots were compared with pure cytochrome *c* and with the spectra of cytochrome oxidase (here also called cytochrome *a*) and cytochrome *b* published by biochemists. Several hundred spectrograms were recorded from vigorously growing wheat roots (only the lower *c.* 60 mm. were used; the roots were divided in sections of 20 mm. length; some 300 such root pieces were tightly packed in a 15 mm. quartz tube provided with inlet and outlet for the solutions).

The wheat roots have a complete cytochrome system. The prosthetic groups are identical with or very similar to those of the cytochrome oxidase and the cytochromes *c* and *b* known from animal preparations, yeast, etc. The presence of cytochrome oxidase was also demonstrated from the oxidation of added reduced cytochrome *c* by living or homogenized roots. The kinetics of the cytochrome system of wheat roots is very similar to that of the heart muscle, both in respect of the turn-over number of the cytochrome oxidase, the state of oxidation of the operating system and its linkage to succinic dehydrogenase (*dh*).

Because the molar extinction coefficients of the different cytochromes calculated per mol. Fe are fairly alike, the approximate percentage of the single enzymes may be calculated from the absorption spectrum. Vigorously growing wheat roots contain on an average 0·84μmol. cytochrome oxidase (a value corroborated by determinations of the oxidation of added cytochrome *c*), 1·56μmol. cytochrome *c*, and 2·46μmol. cytochrome *b* per kg. fresh weight. This corresponds to 21·6 mg. $\times$ kg.$^{-1}$ fresh weight, or 237 mg. $\times$ kg.$^{-1}$ dry weight in respect of cytochrome *c*, values corresponding to quantities found in animal tissue. Cytochrome *b* is, however, more dominant than in most animal tissues.

The cytochromes are approximately uniformly distributed in the lower 60–100 mm. of the roots, a fact coinciding with the earlier established uniform distribution of the power of salt accumulation (Lundegårdh, 1949*b*). A cytochrome system is also present in the green leaves and the coleoptiles of wheat. Roots of rye and maize behave similarly.

XI. CO-VARIATION OF THE ABSORPTION OF ANIONS AND THE STATE OF OXIDATION OF THE CYTOCHROMES

If the presence of movable anions is facilitating the electron transference through the cytochrome system to the oxygen an increasing anion respiration will be accompanied by an increase of the quotient oxidized cytochrome/reduced cytochrome. A co-variation of the active absorption of anions and the value of this quotient must strongly support the hypothesis of a direct causal linkage between electron transference and anion transport.

A close study of the time course of the reoxidation of the completely reduced cytochrome system of the roots (Lundegårdh, 1953*b*) confirmed the conclusion that the electrons are transferred in the following sequence:

$$\text{succinate} \xrightarrow{1} dh \xrightarrow{2} b \xrightarrow{3} c \xrightarrow{4} a \xrightarrow{5} \mathrm{O}_2. \tag{10}$$

The dehydrogenase *dh* is believed to have a band at 570–575 mμ when reduced (Lundegårdh, 1952, 1953). At the start of reoxidation the cytochrome oxidase is immediately oxidized by the oxygen, cytochrome *c* is then oxidized by the oxidase, and so on. A steady state is finally attained, in which the degree of reduction of each member corresponds to the prevailing conditions of the whole system and the potential states of the single members. Under conditions of optimal anion respiration (0·05 mol. aerated salt solution) and at 18–20° C. the cytochromes are predominantly oxidized (60–84 %, cytochrome *b* leading). This means that the first link in the chain, viz. the splitting up of one hydrogen atom uncoupled from succinate in the reaction

$$\text{Succinate} \rightleftharpoons \text{fumarate} + 2\mathrm{H}^+ + 2e, \tag{11}$$

acts as a brake, resulting in a comparatively low state of reduction of cytochrome *b* and the subsequent links of the chain.

The influence of neutral salts on the oxidation-reduction balance of the cytochrome system may be studied by recording the changes in the absorption spectrum occurring at a change of the medium from distilled water to a salt solution, provided the roots were previously desalted. A few seconds after the contact with the salt the reduction peaks (in the oxidation-reduction spectrum) of the cytochromes are visibly lowered (Fig. 4). The observed time-course of the increasing oxidation coincides with the time-course of reoxidation from anaerobic to aerobic in distilled water (Fig. 5), and it also coincides with the time-course of absorption of anions, as observed in parallel experiments with the same material and at the same temperature.

In a series of experiments with the addition of 0·05 mol. KCl the oxidation $\left(=\text{quotient } \dfrac{\text{oxidized cytochrome}}{\text{total cytochrome}} \times 100\right)$ rose from 48% in aerated distilled water to 69% in the areated salt solution for cytochrome oxidase, and from 61 to 84% for cytochrome *b*. The higher oxidation of the latter reflects its lower oxidation potential state; a comparatively small rise of the oxidation level of a cytochrome of high potential will secure a comparatively large oxidation of a cytochrome of lower potential. The response of the

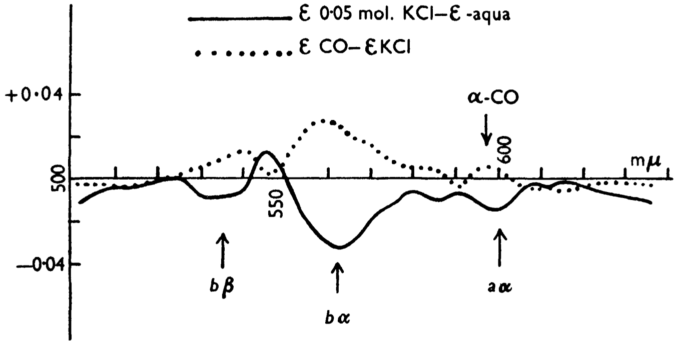


Fig. 4. Oxidation-reduction spectrum of the cytochrome system of wheat roots. Black curve: difference spectrum between roots in 0·05 mol. KCl and roots in aqua, showing the decrease of the reduced bands of the cytochromes *a* (cytochrome oxidase) and *b*. Dotted curve: reduction spectrum in carbon monoxide.

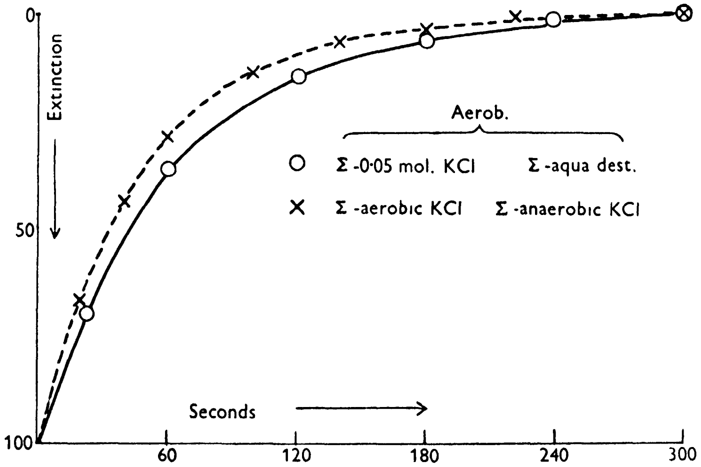


Fig. 5. Time course of oxidation, measured as decrease of the α-band of cytochrome *b*, at a shift from anaerobic to aerobic (dotted curve), and at a shift from aqua dest. to 0·05 mol. KCl. The two processes take an approximately identical course.

cytochrome system to salts and other conditions changing the state of oxidation may be observed on the Soret bands in the violet (γ-bands) or on the bands in the green (α-bands), the former showing a 3–5 times higher extinction in the oxidation-reduction spectrum than the latter. In the case of cytochrome *c*, the α-band at 550 mμ is to be preferred, because the appearance of changes of the γ-band at 418 mμ (oxidation-reduction) is frequently delayed, probably owing to molecular complex reactions (Lundegårdh, 1953*d*). The values of the response of cytochrome *c* to salts (Lundegårdh, 1953*a*, table 3) are for this reason too low. The α-band at 550 mμ shows a very marked response to changing conditions of oxidation (see Lundegårdh, 1953*c*, fig. 2).

Considering the fact that the response of the roots to salts comprises only about one-quarter of the total anion respiration (see above on d.w.-respiration) the observed changes of the oxidation-reduction state of the cytochromes are fairly large. In one experiment with a 10 mm. thick bundle of desalted roots (see Lundegårdh, 1952, p. 491) the height of the Soret band of the cytochrome oxidase was 0·040 ($=\log I_0/I_1$). The addition of 0·02 mol. KCl lowered the value to 0·021. The final addition of 0·001 mol. HCN raised the value to 0·066. The effect of the salt thus amounted to 30% of the amplitude of oxidation-reduction between the state of oxidation in aerated distilled water and complete reduction in HCN. A simple calculation teaches us that the cytochrome system would probably be completely reduced if the 'native anions' could also be removed.

The idling caused by the native anions (organic acids plus the circulating fraction of previously stored salts) is reflected in the state of oxidation-reduction of the cytochrome system if the state of approximately 100% oxidation obtained after treatment with malonate or fluoride (and not the situation in distilled water) is chosen as zero value (see Lundegårdh, 1953*a*). The values of the oxidation in distilled water just mentioned (48–61%) were calculated from the absolute zero value of reduction (100% oxidation). In the case mentioned the increase in oxidation of cytochrome oxidase caused by salt amounted to $69-48=21\%$. The corresponding value of cytochrome *b* was $84-61=23\%$ increase of the oxidation. These values are in good agreement with the conclusion drawn from absorption experiments, that the active salt absorption of wheat roots on an average corresponds to 25% of the total electron transference.

XII. THE ANIONS AS COENZYMES

A further analysis of the effect of salts on the cytochrome system reveals two partial effects or phases:

(1) The exchange of electrons and anions, an electrochemical balancing

process controlling the velocity of turn-over of the enzymes according to the scheme:

$$Ct_{\mathrm{red.}} + \mathrm{anion} \rightleftharpoons Ct_{\mathrm{ox.}}.\mathrm{anion} + e. \qquad (12)$$

Phase 1 represents the *fundamental coenzymatic effect of anions.*

(2) The coenzymatic effect may be developed into a physiological process, the active transport and accumulation of salts, if the *structural arrangement of the cytochromes turns over the coenzymatic attraction of anions into a polar stream* from the medium to the interior of the cell, or from one point of a cell to another point, from which a rapid backflow of salt is prevented by a *structural barrier.*

This analysis of the salt effect explains why cytochrome systems may, in many cases, e.g. in animal cells, operate without any salt accumulation to speak of. These cells apparently lack an organization suitable for salt accumulation. It is, on the other hand, experimentally possible to separate the phases 1 and 2 in objects showing both.

In homogenized root tissue the aerobic respiration slowly decreases, probably owing to an uncoupling of the succinic dehydrogenase, and the power of salt accumulation is lost. The cytochrome oxidase and probably also the other cytochromes, however, maintain their activity several hours after homogenization. In a series of experiments (Lundegårdh, 1953*c*) reduced cytochrome *c* was added to homogenate and the activity of the cytochrome oxidase determined from the disappearance of the band at 550 mμ. Controls were made with addition of cyanide. The activity of the oxidase is largely dependent on the presence or absence of salt ions. If desalted roots are homogenized in distilled water the activity of the cytochrome oxidase was increased 60% after addition of 0·1 mol. KCl or KNO_3. Similar results were yielded by roots from nutrient solutions if they were homogenized together with anion-absorbing resin. Also in this case the addition of a surplus of salts considerably accelerated the activity of the cytochrome oxidase. It was calculated that the abridged chain $c \rightarrow a \rightarrow O_2$ is about as active as in intact roots.

XIII. THE DIFFUSION BARRIER AND CYTOCHROME *b*

Uncoupling of phase 2 may also be brought about by treatment of the roots with dinitrophenol (DNP) or fluoride. Experiments by Stenlid (1950) and Robertson & Wilkins (1948) have shown that DNP in certain concentrations stops the salt absorption in spite of the fact that the cyanide sensitive respiration continues.

Stenlid showed that DNP in some way severely disturbs the cytochrome system, causing a qualitative change in the respiratory processes. Similar effects were observed under the influence of methylene blue. My own experiments with DNP have corroborated the observations of Robertson and Stenlid and show a rapid decline of the active chloride absorption in concentrations of DNP higher than 10^{-6} mol. (at pH *c.* 5; at lower concentrations the normal anion respiration was slightly stimulated), whereas the cyanide-sensitive respiration was even stronger than in the controls up to a concentration of *c.* 3×10^{-5} mol. DNP. The simultaneously observed absorption spectrum of the roots showed a quite normal behaviour of the cytochromes *a* and *c*, whereas cytochrome *b* was obviously more or less put out of action.

Fluoride is a more effective and at the same time more indulgent inhibitor of cytochrome *b*. At a pH promoting the absorption of molecular NaF (*c.* 3·3–3·4; see Lundegårdh, 1949*a*) cytochrome *b* remains completely oxidized whereas *a* and *c* continue their enzymatic activity in connexion with a second dehydrogenase system, the succinic dehydrogenase being put out of action because it cannot transfer electrons to cytochrome *b* if fluoride is present. The second dehydrogenase system is possibly linked to cozymase and a flavoprotein. At inhibited activity of cytochrome *b* this second dehydrogenase system conducts a part of the end-oxidation via cytochrome *c* and cytochrome oxidase. With still active succinic dehydrogenase the second system is probably more exclusively served by a second oxidase system, possibly flavoprotein and/or a non-ferrous metalloprotein (see Fig. 6).

These recent results (see Lundegårdh, 1953*a*) define the question as to the organization of the cytochrome system as a body promoting a polar stream of anions. The theory of anion respiration as it has been elaborated in my later work (1945–53) postulates the cytochromes as carriers of the anions and attributes the accumulation work to the liberation of the anions at cytochrome *b* (1 or 2 in scheme (10)), this place of release of the anions from their carriers obviously being structurally arranged to prevent a rapid back-flow of the anions to the point of absorption (at the cytochrome *a* or 5 in scheme (10)). This theory introduces a minimum of additional hypotheses and is a logical development of the co-enzymatic action of movable anions.

It was stated that phase 1, or the co-enzymatic action of movable anions in the surroundings of the cytochrome system, is probably of universal importance. Few living cells are, however, completely deprived of salts. Moreover, movable anions of organic acids are produced in the stages of aerobic respiration preceding the end-oxidation. The experiments with desalted roots and roots held under conditions lowering the production of organic acids (Lundegårdh, 1949*a*), like desalted slices of storage tissue

(Robertson and his group), clearly demonstrate the existence and physiological importance of the co-enzymatic function of anions.

If phase I means an exchange between anions and electrons the movement of the former may be characterized as an 'active transfer', but in the absence of structural barriers this active transfer will be unable to accomplish a real salt accumulation, because the released anions will find their way back to the starting point by means of normal diffusion. The intermittent function of the cytochromes as anion carriers (in the moments of oxidation) may possibly result in an attraction sphere of anions around the system, a circumstance possibly conveying certain biochemical consequences.

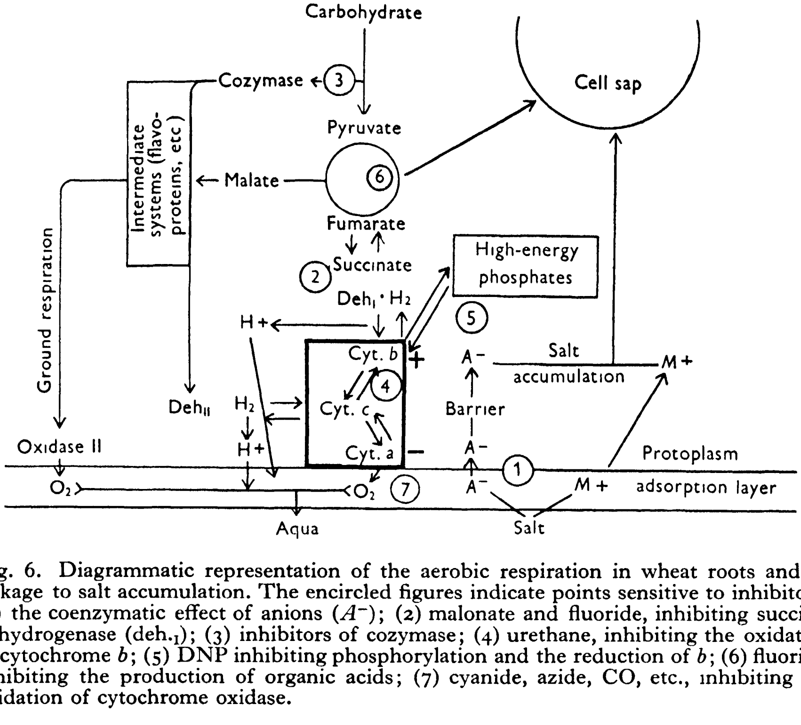


Fig. 6. Diagrammatic representation of the aerobic respiration in wheat roots and its linkage to salt accumulation. The encircled figures indicate points sensitive to inhibitors: (1) the coenzymatic effect of anions (A^-); (2) malonate and fluoride, inhibiting succinic dehydrogenase (deh.$_{\mathrm{I}}$); (3) inhibitors of cozymase; (4) urethane, inhibiting the oxidation of cytochrome *b*; (5) DNP inhibiting phosphorylation and the reduction of *b*; (6) fluoride, inhibiting the production of organic acids; (7) cyanide, azide, CO, etc., inhibiting the oxidation of cytochrome oxidase.

Accumulating evidence supports the assumption that mitochondria are the site of the complete cytochrome system of animal cells and possibly of certain plant cells too. Most animal cells do not accumulate salts to any considerable extent in spite of the dominance of the cytochrome system as respiratory mechanism. The mitochondria do not seem to have any pronounced function as salt accumulators. They may have a membrane (see Farrant, Robertson & Wilkins, 1953), but are apparently not built for the development of a high osmotic pressure. But they may, of course, be figured serving as carriers of an adsorbed layer of salt ions, thus transporting salts from one point of the cell to another by means of protoplasmic streaming or a more independent mobility of the mitochondria themselves.

The assumption of mitochondria as carriers of salts between the surface of the plant cell to the sap space would possibly meet difficulties in explaining the observed quantitative relations between the electron activity and the transported anions. At the present state of knowledge it seems more appropriate to assume a structural linkage of the cytochrome system to the protoplasmic membrane in cells endowed with the power of an effective salt accumulation. This assumption is, moreover, supported by the real presence of cytochrome oxidase in the surface of the roots (Lundegårdh, 1952, 1953). The kinetics of the cytochrome system (Lundegårdh, 1953*b*, *c*) leaves little room for ideas about a local separation of the cytochromes, e.g. the oxidase placed in the surface and cytochrome *b* at the vacuole membrane. The cytochromes are very probably joined to a structural body in which, however, the single enzymes have free thermal mobility (cf. Fig. 7). Under these circumstances the loci of absorption and accumulation of salts lie within a distance of only *c.* 150–200 Å. (see below),

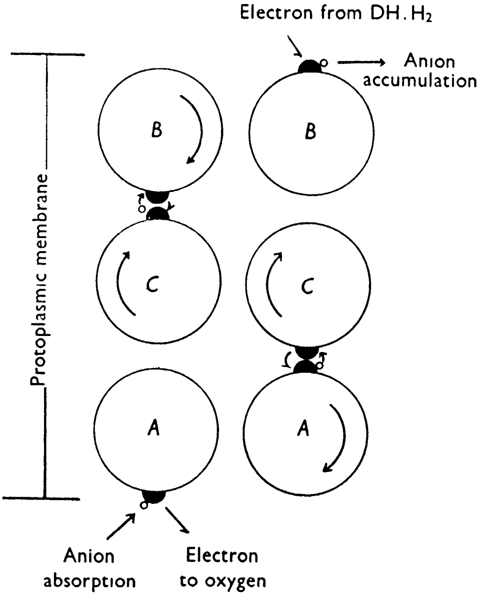


Fig. 7. Scheme of the coenzymatic action of anions and their transport from the point of absorption to the point of accumulation. The large circles symbolize the apoenzymes (*c* is actually smaller than *a* and *b*), the black semicircles the coenzymes.

or on both sides of a membrane. The original place of salt accumulation will then be the protoplasm. As previously mentioned, a free-exchange equilibrium probably exists between the protoplasm and the cell sap and there is little experimental support for assuming an active secretion of salts through the vacuole membrane.

A simplified scheme of the transference of electrons and anions is shown in Fig. 7 and in the following sequence of reactions:

Absorption

1. Cytochrome oxidase (Fe^{2+}) + *anion* + $\frac{1}{4}O_2 \rightarrow$ cytochrome oxidase $(Fe^{2+})^+$.anion + $(\frac{1}{4}O_2)^-$.
2. Cytochrome oxidase $(Fe^{2+})^+$.anion + cytochrome *c* $(Fe^{2+}) \rightleftharpoons$ cytochrome oxidase (Fe^{2+}) + cytochrome *c* $(Fe^{2+})^+$.anion.
3. Cytochrome *c* $(Fe^{2+})^+$.anion + cytochrome *b* $(Fe^{2+}) \rightleftharpoons$ cytochrome *c* (Fe^{2+}) + cytochrome *b* $(Fe^{2+})^+$.anion.
4. Cytochrome *b* $(Fe^{2+})^+$.anion + $\frac{1}{2}$ DH.$H_2 \rightleftharpoons$ cytochrome *b* (Fe^{2+}) + $\frac{1}{2}$ DH + H^+ + *anion*.

Accumulation

(13)

The molecular construction of the haemin groups is still incompletely known (see Lemberg & Legge, 1949). It may be sufficient to symbolize the loss or gain of one electron as a change of the net charge of the molecule. The cleavage of H into $H^+ + e$ marks the end-stage of accumulation. The postulated diffusion barrier must be situated somewhere between stage (1)–(2) and the rest of the electron ladder because it was experimentally shown that the active accumulation stops if cytochrome *b* only is inactivated (see above). The barrier may simply be regarded as retarding diffusion of anions through the membrane structure. The activating effect exerted by the cytochrome system will then move the anions preferably in a centripetal direction, the stages (5) and (1)–(2) of the scheme (10) respectively providing the energy for the entrance and exit of the anions.

According to reaction (4) the accumulated anions are combined with an equivalent quantity of hydrogen ions, A^- and H^+ together forming a strong acid, but this acid immediately reacts with the cation carriers $M^+.R^-$, as a result of which preferably neutral salts will be accumulated. As an equivalent quantity of protons are consumed in the reaction

$$(5)\quad (\tfrac{1}{4}O_2)^- + H^+ = \tfrac{1}{2}H_2O, \qquad (13a)$$

the accumulation of a neutral salt does not change the cH balance.

XIV. ACTIVE TRANSPORT OF CATIONS

If the anion respiration is considered as an electrochemical phenomenon, implying the transference of electricity from one point to another, the whole process may also be pictured as an electrophoresis between the positive pole of the system, represented by cytochrome *b*, and the negative pole, represented by cytochrome oxidase. The streaming of anions may from this viewpoint be interpreted as a regular electrophoresis between the poles of an electric battery (Figs. 6, 8). The electron transference between the single cytochromes corresponds to the internal transference of electrons proceeding in a battery. This scheme opens certain new aspects as to *an active transport of metallic cations.*

Electrophoresis comprises a streaming of an equivalent quantity of cations in a direction opposite to that of the anions, in our case from the region of the dehydrogenase system to the surface boundary. This reversed stream would imply a continuous loss of metallic cations if a corresponding quantity of protons were not produced at the positive pole and consumed at the negative pole (reaction (5)). The net result will then be a one-sided transport of anions (Fig. 8A). If, however, the protons produced at the positive pole are partly consumed in an excretion of the free acid HA—a process implying an acidification at the point of accumulation—metallic cations will be caught by the centrifugal stream of positive electricity (Fig. 8C), and we have a respiratory mechanism excreting cations.

An excess of metallic cations (K, Ca, Na, etc.) may exist after periods of abundant salt absorption concomitant with internal consumption of anions, especially nitrate of which *c.* 50% is normally proteinized in the wheat roots (Burström, 1939). An excess of metallic cations may also result from an abundant production and storage in the cell sap of organic acids followed by a period of their metabolic consumption. As shown by Ulrich (1942) and Burström (1943) absorbed cations and produced organic acids—primarily malic acid—are important factors in maintaining a constant internal pH. During metabolic consumption of organic acids the supernumerary cations may be returned to the medium partly by non-metabolic exchange, partly by means of active excretion in the anion respiration mechanism (Fig. 8B). Theoretically a situation may be figured in which no anions are actively absorbed from the medium, only organic acids metabolically produced. Given a previous abundant storage of metallic cations in the cell the anion respiration will at first sight behave exclusively as a mechanism for the excretion of cations. Anions acting as co-enzymes are in this case OH^-, HCO_3^-, and organic acids. The participation of the cytochrome system in an active transport of cations has been very little studied, but the problem certainly deserves more attention.

The electrochemical properties of the complete cytochrome system are well fitted for a separation of anions and cations of a salt on the two sides of a membrane barrier. Examples of this property are the excretion of chloride from the gastric mucosae, and the strong acidification of the cell sap in certain plants.

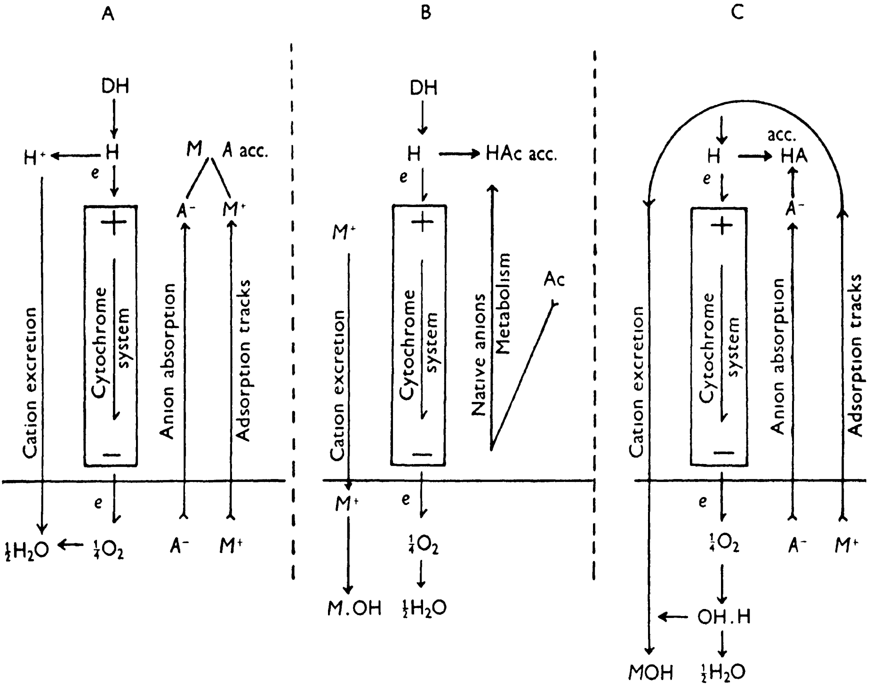


Fig. 8. Diagram illustrating the electrophoretic activity of the enzyme body of the cytochrome system and its activity as a mechanism for absorbing anions and excreting cations. See the text.

XV. THE LOCALIZATION OF THE CYTOCHROME SYSTEM IN THE CELL

The probable localization of the cytochrome system of wheat roots at the cell surface is supported by the observation of an oxidation of added reduced cytochrome *c* by living roots (Lundegårdh, 1953). Wheat roots in good health contain 2·56 μmol. cytochrome $b \times \text{kg.}^{-1}$ (fresh weight), or $2{\cdot}5 \times 10^{-3}$ μmol. $\times \text{ml.}^{-1}$. At a diameter of 0·5 mm., 1 ml. tightly packed roots have a total surface of 63 cm.^{2} or 63×10^{16} Å.^{2}. The total surface of all cortex cells is approximately 8×10^{19} Å.^{2}. At a number of 6×10^{23} molecules in 1 mol., 1 ml. root tissue thus holds 15×10^{14} mol. of cytochrome *b*, viz.

only one molecule on a surface of *c.* 5 × 10⁴ Å.², the mean distance between two molecules amounting to *c.* 220 Å. The molecular weight is known only for cytochrome *c* (13,000) and is calculated to be 75,000–80,000 for cytochrome oxidase (Warburg, 1946). Assuming the molecular weight of cytochrome *b* to equal that of the oxidase its diameter, at close packing of the atoms, would amount to 35–40 Å. At a mean distance of 220 Å. cytochrome *b* would then be conveniently placed in a single surface layer (of the dimensions of membranes of mitochondria and other structures, see Sjöstrand, 1953). It was earlier calculated (Lundegårdh, 1940) that the mean distance of the cation carriers in the root surface is 160–170 Å., a value of similar magnitude to the above. Owing to the lower concentration, a surface layer of cytochrome oxidase would, however, show a more spacious distribution, resulting in a surface concentration amounting to only about one-third of the concentration of the cation carriers. Because the molecules of the cytochrome oxidase according to the theory are serving as anion carriers (R^+), the calculated figures are well in accord with the actually shown predominance of the R^- groups (see above). From what is known about the kinetics of the cytochrome system (Lundegårdh, 1953*b*) the three cytochromes are probably comparatively tightly packed in groups of 1*a*, 2*c*, and 3–4*b*. Even the distribution of such groups in one single layer in the surface of the cell would leave ample space for other constituents of a complex membrane of mosaic pattern, possibly also including other enzyme systems.

XVI. DISCUSSION

Considering the outstanding importance of an active transport of salts and other solutes into cells and between cells of a tissue or organism, the number of investigators devoted to these problems is still very limited. And the single investigators mostly reveal a keen ambition to present new theoretical schemes. It is a remarkable fact, to which I have previously alluded (Lundegårdh, 1940, 1949*d*, p. 324), that workers in the field of 'diffusion permeability' ignore the results on salt accumulation in spite of the fact that the existence of an ionic activity of the protoplasmic membrane certainly interferes with the properties of passive permeability on interaction between sugar absorption and ions (see Lundegårdh, 1940; Lundegårdh & Burström, 1944 and other papers). It is, furthermore, known that passive diffusion is not the simple line of communication between a cell and its surroundings once believed, because a number of common nonelectrolytes, e.g. sugar, asparagine, etc., are actively transported and still obscure manifestations of 'active transport are involved in transport mechanisms in sieve tubes or along cell surfaces in parenchymatic tissue.

Investigations on anion respiration and related problems inevitably include biochemical work. The discovery of the co-enzymatic effect of anions on the cytochrome system has, however, evoked no rejoinder from the side of the biochemists. Chance (1952, 1953) overlooks this effect in his recent attempts to understand the reaction kinetics of the cytochrome system. The research work on the cytochrome system of wheat roots, presented here, illustrates the usefulness of suitably chosen and treated living material as objects for biochemical work. It has been shown that the living cells are surprisingly permeable to all biochemical inhibitors, if attention is paid to the conditions of dissociation (Lundegårdh, 1949*a*; Stenlid, 1950). A co-enzymatic effect of anions was recently observed in the photosynthesis of isolated chloroplasts (Gorham & Glendenning, 1952). The attempts of these authors to explain the results from obscure effects of ions on 'colloidal properties' are, however, futile. Cytochrome is present in the chloroplasts (Hill, 1951) and a co-enzymatic effect of anions is thus feasible, but anions may of course also operate as balancing agents in other processes of electron transference than redox reactions of cytochromes.

Certain authors refuse to discuss the theory of anion respiration because they think it is not 'universally accepted' (Preston, 1948, p. 130), and recently Overstreet & Jacobson (1952) failed to recognize what the theory really contributes, owing to an incomplete knowledge of various papers in which it was experimentally elaborated. The critical attitude of Steward (1935 and later papers) is unique, because he simply denies the existence of any evidence of a quantitative relation between an anion or salt respiration and the absorption of ions. In fact, Robertson and his group in a series of papers clearly demonstrated the existence of an anion (or salt) respiration in the same material that Steward used.

Besides this negative attitude, Steward also presents his own theory on salt accumulation, namely, an assumed linkage to growth and protein metabolism (Steward & Preston, 1941). I have previously shown (1945, p. 31) that the extremely active cytochrome system is obviously linked to various synthetic processes, the synthesis of proteins among others. The active transport of salts is by no means the main purpose of the cytochrome system, but merely an accessory process consuming a very small fraction of the converted energy (see p. 281). We know at present that the main part of the energy of respiration is stored in high-energy phosphate bonds of which the protoplasm disposes for special purposes. The active accumulation of salts thus runs simultaneously with the uphill side of the complicated steady-state situation characteristic of living cells.

In the case of wheat roots it is known that active growth is restricted to a tip zone of a few millimetres length. The nitrate assimilation, supporting the synthesis of proteins in the root (Burström, 1943; Lundegårdh, 1945, 1951*a*), is extended over the lower 20–30 mm. of the root. In this zone only, *c.* 50% of the absorbed nitrate is consumed and utilized for synthetic work. In the zones above 30 mm. up to 100 mm. from the tip, 100% of the absorbed nitrate is exuded in the sap stream (Lundegårdh, 1951*a*). The cytochrome system and the power of active salt absorption is, however, uniformly distributed over the whole length of *c.* 100 mm. (Lundegårdh, 1949*b*, 1952, 1953*c*). Also experiments on the effect of a number of inhibitors on growth and salt absorption illustrate the lack of parallelism between these processes. The same conclusion is drawn from experiments with indole acetate (IA), the results of which are plotted in Fig. 9. Owing to a complex effect of the hormone and the H-ions (see Lundegårdh, 1949*d*) the rapid growth reaction (30 min.) varies along a reversed optimum curve, the increasing retardation dominating up to a concentration of *c.* 10^{-5} mol. IA and a pH (caused by the partial dissociation) of *c.* 4·9, followed by an increasing stimulation in the concentrations 10^{-4} to 10^{-3} mol. IA and pH values 4·4 to 3·9. The respiration and the absorption of added chloride (unpublished experiments) give curves of a different pattern, the chloride absorption remaining intact up to 10^{-6} mol. IA (at a growth inhibition down to 30%) and after a rapidly passing stimulation at 10^{-5} IA sinking to 45%

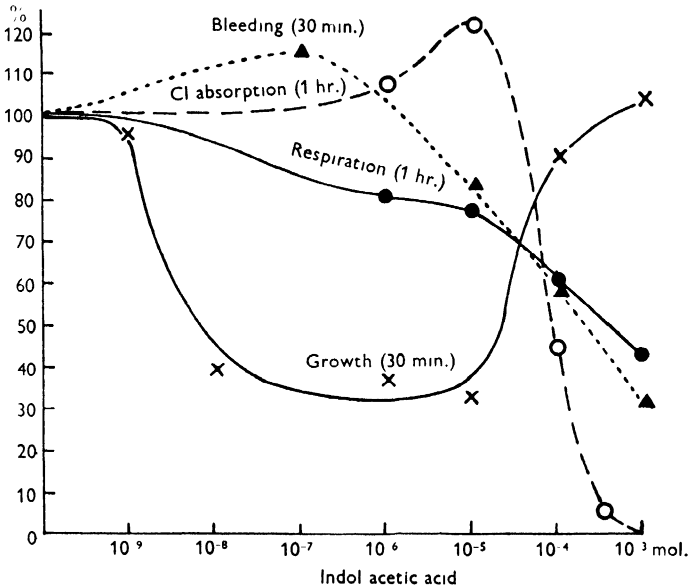


Fig. 9. Diagrammatic representation of experimental series on the effect of indole acetic acid on growth, bleeding, respiration, and Cl absorption.

in 10^{-4} and 5% in 10^{-3} mol. IA. The total respiration decreases slowly, followed by the curve of bleeding (gross volume of the exuded sap). Separate determinations of the respiration show disturbances both in Q an./O_2 and the ground respiration.

There is consequently ample experimental evidence of a full activity of the cytochrome system in cells of inhibited growth and of minimal protein synthesis. The cells 100 mm. from the tip of rapidly growing grass roots are of course comparatively 'young', and it is possible or even probable that in older parts both the cytochrome system and the power of active salt absorption disappear.

Humphries (1951, 1952), conducted experiments with roots of barley and pea from which he concluded that there is 'no evidence of a salt respiration'. As Humphries made no experiments with cyanide he apparently knows very little of anion respiration in his objects. Also his discussions reveal lack of knowledge of the vast experimental material on which the theory of anion respiration is founded. Humphries advances speculations as to the sugar level of the cells as a promoter of salt accumulation. The hypothesis that sugar 'may be the parent substance for the formation of a chemical compound capable of combining with ions' has no real meaning because sugar participates in a multitude of biochemical processes and is 'the parent substance' of nearly everything in the protoplasm. In my experiments the role of sugar as the fuel for the anion respiration was quantitatively demonstrated. At low sugar level the anion respiration may be retarded. It then accelerates after sugar has been supplied. Unpublished experiments show that sugar supplied simultaneously with chloride stimulates the absorption of anions, whereas roots observed after a preceding period of feeding with sugar reveal a slight retardation of the anion absorption. It was previously observed that feeding with sugar raises the level of acidity in the cell sap. As shown above, organic acids compete with inorganic anions in the anion respiration. Sugar is absorbed into the roots by means of an active process (Lundegårdh & Burström, 1944), probably respiratory phosphorylation. In this activated state the sugar is probably more accessible for the anion respiration mechanism.

Humphries has observed that salts may be absorbed without any appreciable change in the total respiration. This observation is neither new nor is it surprising. The analysis of the d.w.-respiration teaches us that roots can show a considerable anion respiration if sufficient 'native anions' are present. If such roots are transferred to a salt solution the newly imported anions slowly take over the co-enzymatic function of a corresponding number of native anions. During this competition process the intensity of the anion respiration is but little changed. But it is continuously working, as may be seen from the inhibition by cyanide or from a direct observation of the cytochrome system. Only if the objects are washed in aerated distilled water of a pH somewhat below 6·5 during a sufficiently long period do the native anions disappear to a degree permitting accurate measurements of the quantitative relations between absorbed anions and the anion respiration. One cannot expect that a living tissue will expose the results of one single physiological phenomenon unless a number of interfering reactions are slowed down to a minimum. In long-term experiments with intact plants the passive transport of salts due to respiration has to be considered. As previously mentioned, the exchange capacity (or 'free space') of the cells provides the plant with an instrument of passive translocation of salts, the extension of which, however, can be determined only from careful studies of the simultaneous active anion respiration. Such an analysis has been omitted in the studies of Humphries and in the more recent work of Hylmö (1953).

The investigations and discussions of Robertson & Wilkins (1948) as to the effect of DNP has awakened doubts on the validity of the theory of anion respiration also among other writers. Overstreet & Jacobson (1952, p. 202) venture that 'it is rather difficult to fit the DNP effect into the Lundegårdh hypothesis, as presently postulated, without some further assumptions or extensive modification'. It was shown in the preceding pages that no 'extensive modifications' are needed. The key position of cytochrome *b* plus *dh* elucidates the problem.

The observations by Stenlid, Robertson & Wilkins and Lundegårdh that DNP also promotes an exuberant exudation of organic substances from the tissues points to a severe disturbance of the structural qualities of the protoplasmic membrane. It has been assumed that the succinic dehydrogenase is involved in the high-energy phosphate ($=\sim$ph) metabolism (Schlenk, 1951). A tentative scheme is the following:

$$\text{Succinate} + 2\text{Fe}^{3+} + \text{ADP} + \text{PO}_4 \rightleftharpoons \text{Fumarate} + 2\text{Fe}^{2+} + 2\text{H}^+ + \text{ATP}. \quad (14)$$

The balance will be moved to the right in the presence of predominantly oxidized dehydrogenase, a situation realized in a respiring cell given a sufficient supply of salts. At very high concentration of fumarate and predominantly reduced cytochrome *b* the equilibrium will move to the left side, a situation observed in living roots after addition of fumarate + HCN (Lundegårdh, 1953). According to the formula $\Delta F = -n\text{F}\Delta E$, where ΔF = free energy, F = Faraday or 23,000 cal., n = the number of electrons and E = the potential gap (Kaplan, 1951, p. 64), one $\sim$ph will be synthesized at $E = 0{\cdot}3$ V. if two electrons are participating in the equilibrium. These facts are concordant with the calculated relatively high oxidation-reduction potential state of cytochrome *b* (Lundegårdh, 1953*a*). It is a common belief that ATP is an important guard of the structural integrity of the protoplasm, and the key position of cytochrome *b* in the anion respiration may also be considered from this viewpoint. Fluoride inhibits the succinic dehydrogenase and enzymes participating in the production of organic acids, but not phosphorylation. The similar effects of DNP and fluoride on the anion respiration (Lundegårdh, 1952, 1953) points to the *inhibition of the electron transference* from dehydrogenase to cytochrome *b* as the immediate cause of inhibited accumulation of anions.

As to the suggestion of Robertson & Wilkins, that the ~ph metabolism might be directly involved in accumulation work too, this possibility was already discussed in connexion with other possible mechanisms of salt accumulation. Because values of Q an./O_2 above 4 were not observed in plant material there is so far no need for any other mechanism than the anion respiration, if a cytochrome system is at work. Speculations as to variations in potential gradients, viz. the acid/base balance (Helder, 1952; Vervelde, 1952), or in the dissociation of ion carriers (p. 264), must be supported by quantitative experimental investigations before the matter can be taken under consideration. Helder (1952, p. 421) ventures as an argument against the carrier function of the cytochromes that the ratio of the absorbed quantities of different anions 'is quite different from that of the composition of the external solution'. This is a poor argument because (1) the cytochromes, as other ion carriers, may of course show selective qualities, and (2) there are also other anion carriers in the surface of the cell, with which the cytochromes may exchange anions. If the latter act selectively the total absorption will be selective.

A few words may finally be said about electrical currents in salt-absorbing organs. Roots produce an electrical current continuously if a circuit is closed between the tip and the base (Lundegårdh, 1940). If the current is accelerated by the addition of a 2 V. battery the anion respiration and the salt absorption are also accelerated. If the extra current flows in the opposite direction salt absorption and respiration are retarded. These results are quite in accord with the assumption that the stream of electrons through the cytochrome system is the source of electricity. But they are not quite conclusive because electricity will be produced in connexion with every active transport of ions. An operating cytochrome system augments the electron density in the organ, hence lowers its ohmic resistance. Unpublished measurements show an increase of the conductivity at full activity of the cytochrome system and a decrease after the addition of cyanide. But also the production of organic acids has to be considered here. Measurements of bioelectric potentials and bioelectric production of electricity may reveal important physico-chemical properties of the cells, as shown in the discussion of root potentials. Reliable conclusions as to the mechanism of salt accumulation are, however, attained only from a biochemical and biophysical analysis of the phenomenon *in vitro* and *in vivo*.

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SOME ASPECTS OF ION TRANSPORT THROUGH MEMBRANES

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I. INTRODUCTION

In the following article, work relating to active transport of sodium and potassium ions in yeast and some new evidence relating to the localization of sodium in muscle from the author's laboratory are mainly considered, together with some recent work from other laboratories relevant to the 'redox-pump' theory of active transport (Conway, 1951, 1952, 1953). At the outset some questions of a general kind arise, including the nature of the immediate energy source in transport of ions to a higher electrochemical potential. Under this latter heading no general review is attempted, but the 'redox-pump' theory mainly considered (which is dealt with much more fully elsewhere; Conway, 1953), as well as the irreversible energy change in the transport of free ions through membranes.

Active transport across a membrane connotes movement of the solute or ion across the membrane dependent on the activity or energy change of *another* system. Passive transport is equivalent either to free diffusion or to 'exchange diffusion' where the energy of the net movement comes from the *same* system, as where urea diffuses from a higher to a lower concentration across a membrane. Here the system is urea on both sides of the membrane.

Active transport so defined may in turn be considered as equivalent to functional transport, and the following may be considered to exemplify different kinds of functional transport or transference.

(*a*) The solute is removed from free solution by a carrier, the resulting complex then traversing the membrane and yielding the carried solute on the other side by enzyme action. In this the carrier itself crosses the membrane in quantitative or equivalent relation with the carried solute. Lipoid solubility of the carrier complex may be assumed.

(*b*) This is a process similar to (*a*) but with cyclical restoration of the carrier representing what are usually regarded as the most typical cases of *active* transport. Here also lipoid solubility would appear advantageous or necessary. Cyclical activity may be assumed associated with redox changes either directly or indirectly, the latter through conversion of the electron energy into that of phosphate bonds.

(*c*) An ion is brought across the membrane at a certain rate, by a potential difference or gradient produced by the carrier transport and release of an ion of opposite charge. Here the ion itself is passive and the process not usually regarded as one of active transport, and distinguished therefrom; yet in a steady state energy has to be continuously expended upon the maintenance of the potential difference which pulls across the free ions. Such transport, however, may be considered as secondary to 'carrier transport'. (In this context one may consider the question whether an iron filing jumping up to a magnet is 'actively' transported; and if it jumps through a viscous fluid, the question is even more pertinent.)

Clearly, apart from verbalisms, the significant feature is the expenditure of energy by another system in the process, and the energy requirement for the passage of such 'passive' ions is considered later.

(*d*) Functional transport may also be exemplified when a current of fluid is set up through the pores of a membrane as by electro-endosmosis, carrying with it various solutes, and if such are selected, as by molecular diameter and pore size, a certain specificity can be obtained in the solute carriage.

Such a flow of water might also be produced by the special secretion or active transport of one type of solute, with subsequent osmotic flow of water, or again could be produced by the increase of individual molecules or ions through enzyme action on one side of a membrane.

(*e*) Another form of ion passage, which at least arises for consideration, occurs when the chemical potential of a salt, such as potassium phosphate, is lowered on one side of a membrane by metabolic activity incorporating the phosphate ions in organic esters, and a consequent movement of both potassium and phosphate ions to restore the balance (Boyle & Conway, 1941), assuming provisionally that phosphate ions pass as free ions through the membrane. A characteristic of the movement here is that a new equilibrium is reached after the withdrawal of the phosphate ions, and to maintain such equilibrium no additional metabolic activity directed thereto is essential (apart from the slow extrusion of sodium ions).

The transport in such examples (*a*)–(*e*) is effected by special carriers, or by the provision of an electrochemical gradient, or a flow of water or by special metabolic activity. All of these processes depend on activity of some kind and the expenditure of energy by another system, and it may be assumed that all are of functional significance. One may then speak of *active* or *functional transport* as providing the widest heading, divided in turn into *carrier* transport and *free* transport.

Carrier transport may bring an ion to a higher or to a lower electrochemical potential (vide Rosenberg, 1948). If to a lower then the functional significance of the carrier transport would appear to be the *facilitation* of membrane passage. *Free* transport occurs always to a lower electrochemical potential.

In (*c*) and (*e*) above the passage across the membrane is necessarily to a *lower* electrochemical potential, but not necessarily so in (*d*). The reader may also be referred to an interesting discussion of this question from a somewhat different point of view by Linderholm (1952).

II. THE ENERGY SOURCE IN TRANSPORT TO A HIGHER ELECTROCHEMICAL POTENTIAL

When carrier transport of an ion occurs to a higher electrochemical potential, then immediately on release of the ion there must be a simultaneous transference of free energy.

In other words, on the breakdown of the complex, part at least of its chemical energy must appear in the increased electrochemical potential of the transferred ions. Such energy may be directly derived from a redox system transferring electrons, or by the indirect use of such energy as by phosphate bonds.

The use of phosphate bond energy

One way in which bond energy may be used in active transport has been suggested by Danielli (1952), who pictures such energy utilized, as it is in muscular contraction, for the contraction of protein chains on which ions have been loosely combined. Another type of theory may be illustrated by that of Nielsen & Rosenberg (1951) for the secretion of hydrogen ions by the gastric oxyntic cells. (This is referred to again at the end of this article.) Davies & Krebs (1951) also discuss the operation of ATP in the control of the interaction of the ferri-ferrocytochrome and flavine systems.

The 'redox-pump' theory for the active transport of inorganic cations. This has been described elsewhere (Conway, 1951, 1952), and the theory is treated in much greater detail in another publication (Conway, 1953). The following is a brief account.

$$\overline{M} \rightleftharpoons M + e \tag{1}$$

represents a metal respiratory enzyme in the reduced and oxidized condition. E_m, the potential of the system, is independent of the pH.

The equation

$$\tfrac{1}{2}Ct\mathrm{H}_2 \rightleftharpoons \tfrac{1}{2}Ct + \mathrm{H} \rightleftharpoons \tfrac{1}{2}Ct + \mathrm{H}^+ + e \tag{2}$$

represents a system, the potential of which is dependent on the hydrogen-ion concentration. The potential of system (1) is written E_m, and of system (2), E_{Ct} with both systems at a pH, say, of 6·0. It is assumed for convenience in writing that the reductant concentration is the same as the oxidant in both systems. If they are joined by metal electrodes and liquid bridge, and *dn* equivalent electrons allowed to pass from system *M* to system *Ct* then one may write for the free-energy change

$$dn\,\mathrm{F}(E_m - E_{Ct_a}) + dn\,RT\ln(\mathrm{H})_a = dn \times a \text{ constant.} \qquad (3)$$

If the hydrogen-ion concentration of the system is now raised until the potential of system (2) is equal to that of system (1), then, as before,

$$dn(E_m - E_{Ct_b}) + dn\,RT\ln(\mathrm{H})_b = dn \times a \text{ constant,} \qquad (4)$$

and, as the first term is zero,

$$dn\,RT\ln(\mathrm{H})_b = \text{a constant,}$$

then on subtracting equations (3) and (4), then

$$F(E_m - E_{Ct_a}) = RT\ln(\mathrm{H})_b/(\mathrm{H})_a. \qquad (5)$$

This may be interpreted to mean that the whole of the externally available free energy associated with the passage of one equivalent of electrons from system (1) to (2) at a H level of $(\mathrm{H})_a$ can be converted into the energy required to raise one equivalent of hydrogen ions from the level $(\mathrm{H})_a$ to $(\mathrm{H})_b$. The use of such a principle for secreting H ions in high concentration requires a certain organization of enzymes with respect to the cell membrane. This has been treated at length elsewhere as the 'redox theory' for the secretion of H ions (Conway, 1952; vide also Davies, 1951). The evidence for such a theory both for the oxyntic cell of the gastric mucosa and for the yeast cell is very strong.

The secretion of inorganic cations by such a redox-pump system, that is, by direct use of the electron energy, may be treated in an analogous manner.

Thus if one considers the system

$$(\mathrm{Red.}^{-}B^{+}) \rightleftharpoons Ox + e + B^{+}, \qquad (6)$$

in which $(\mathrm{Red.}^{-}B^{+})$ represents an adsorption complex of an inorganic cation with the negatively charged reductant of a respiratory enzyme, *M'*, then, as above, one may deduce that the relation

$$F(E_m - E_{m'_a}) = RT\ln(B)_b/(B)_a$$
$$= \text{a constant.} \qquad (7)$$

(Here, also, both redox systems may be assumed for convenience to have their oxidant and reductant concentrations equal.) Thus at a low concentration of '*B*' $(E_m - E_{m'_a})$ may be assumed to be relatively high, but if '*B*' is progressively increased, then a concentration $(B)_b$ is reached when $(E_m - E_{m'_b})$ approaches zero.

Equation (7) may be interpreted to mean that all the electron energy available from the transference of one equivalent of electrons from one system to the other at a 'B' level of $(B)_a$ can be transformed into the osmotic energy required to raise one equivalent of B from level $(B)_a$ to $(B)_b$. An analogous equation may be written for the active transport of anions, concerning which in root hairs much valuable work has been done by Lundegårdh (1940, 1947, 1948, 1949).

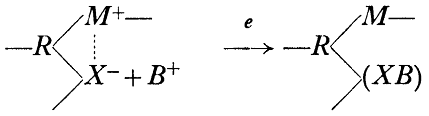
Application of the principle summarized in equations (5) *and* (7). This has been outlined in detail elsewhere (Conway, 1951, 1952, 1953). In the case of the secretion of H ions, metabolic hydrogens are considered to be transferred by way of flavine enzymes to a metal catalyst in the membrane which receives the H atoms, splitting off H^+ ions and retaining the electrons, which are then transferred to oxygen in the case of the oxyntic cell. When the electrons combine with oxygen, H ions are taken up in this last stage from within the cell, in equivalent relation to those produced outside.

With the transport of inorganic cations, in the first stage, the metal catalyst receives H atoms, splits off H ions and retains electrons, the negatively charged catalyst then combining with the inorganic cation in a complex, which passes into the cell membrane. Here, with a final transport of electrons to oxygen within the cell, there is an uptake of H ions, which is equivalent in amount to the H ions liberated therein at the first stage, so that the cell remains neutral.

The question of specificity of carriage

When K^+ ions are carried into the yeast cell or Na^+ ions carried out considerable specificity of carriage is shown. It would appear then that the complex of inorganic cation and reduced respiratory enzyme does not result simply from long-range electrostatic forces.

The following suggestions may be made concerning this point. It may be pointed out that considerable physical differences may occur between certain salts of K and Na. One such salt of an acid may be practically insoluble and the other by comparison very soluble. Also, in the case of certain salts of polyphosphates (Von Wazer & Campanella, 1950), specificity is shown with respect to the degree of ionization. Hodgkin (1951) cites the experiments of Schwarzenbach, Kampitsch & Steiner (1945, 1946) showing that certain organic compounds had a weak affinity for Na but not for K, and evidence from Lamm & Malmgren's experiments (1940) that the polymers of metaphosphoric acid have a special affinity for sodium. It may also be considered that, in general, enzyme action begins with formation of a compound of enzyme with substrate, and often the intimate nature of such a compound is very problematical. In the present context we may speak of a Na enzyme or a K enzyme. In any case, if there are more than electrostatic forces, it would seem that an ionic bond is operative, the question being how such a bond is affected by the redox cycle. The following general possibility may be considered. In the oxidized state of the molecule one may have some such arrangement as



where an attractive force is exercised between a charged atom which suffers a valency change in the redox cycle and a negatively charged atom in another group on the molecule. This force may be sufficient, taking the group of molecules as a whole, to displace Na or K ions wholly or in part from attaching at X, in the manner of an undissociated salt. When the valency change occurs in the M atoms and they gain electrons, the attractive force with X disappears, and Na ions are taken on. In this case the inorganic ion attachments are not directly to the M atoms. (Also it may be noted that such an atom changing its valency in a redox cycle may be nitrogen.)

Kinetic limitations

It has been considered that the full conversion of the electron energy to osmotic work occurs when the donating system and the acceptor systems have the same potential. In such a theoretical case the transfer of electrons would not in fact occur, and if there is a slight difference only it would occur presumably at a relatively slow rate. In such a case, if the metabolic system feeding the electrons or H atoms into the mechanism proceeds more or less unchanged, the donating system will become more and more reduced and the potential difference between it and the accepting system increased.

III. THE SIGNIFICANCE OF TRANSPORT POTENTIALS

Here a distinction may be drawn between assumed and true transport potentials. The assumed transport potential (Ussing & Zerahn, 1951; Teorell, 1952; Linderholm, 1952) has arisen in connexion with the development of flux equations. Thus the ratio of the flux of an ion across a membrane in direction 1–2 to that in the 2–1 direction may be written

$$-n_2^{+\prime}/n_1^{+\prime} = \frac{c_2^{+\prime}\xi}{c_1^{+\prime}}, \tag{8}$$

where $n_2^{+\prime}$ and $n_1^{+\prime}$ are the moles of a cation species transferred, $c_2^{+\prime}$ and $c_1^{+\prime}$ the concentrations on each side, ξ being the Planck symbol $(=e^{EF/RT})$. If active transport is associated with the ion movements, then this ratio does not hold. To equalize the ratios, $c_1^{+\prime}$ could be multiplied by a number α. This equalizing number may then be presented as ξ_t or as $e^{E_tF/RT}$, in which the potential E_t is assumed to be, or called, a transport potential.

Objections to the use of this concept of a transport potential are that, depending on the mobility of the free ions through the membrane, a whole series of figures can be obtained for E_t, even though the active transport mechanism remains the same in kind and in energy output, and even if the number of active carrier molecules remains the same. Also if E_t were regarded as a hypothetical potential which could move the free ions (carried in active transport at the same rate), the energy required for this is quite different from that involved in their actual transport.

These points become clearer when one considers energy requirements in relation to fluxes and transport of ions.

However, there is one value of ξ_t which may be regarded as truly significant when, for instance, as in transport of Na ions through the frog skin, the concentration of NaCl on both sides of the membrane is the same ($c_1^{+\prime} = c_2^{+\prime}$ in equation (10)), and a counter-potential is applied across the membrane sufficient to prevent the active transport of Na ions, then $\xi = \xi_t$ (Ussing & Zerahn, 1951). Here the applied potential gives the maximum potential under the conditions against which the Na^+ ions can be actively transported.

Apart from this it is shown below that a true transport potential for *free* ions is a significant figure. It may be defined as the extra electrical potential required to bring a free ion across a membrane at a given rate. Such a transport potential in turn arises from the carrier system ferrying bound ions of opposite charge.

IV. MINIMAL ENERGY CHANGES INVOLVED IN ACTIVE TRANSPORT

In the following treatment, it will be considered for simplicity that Na^+ ions are being actively transported, that Cl^- ions are being transferred in a secondary way by the resulting potential difference, and, further, that the actively transported Na^+ ions do not diffuse back again through the membrane or, in other words, that the membrane is impermeable to free Na^+ ions.

The classical differential equation (Planck, 1890; Nernst, 1888, 1889) relating the flux of an ion to the electrical and concentration gradients may be written in the form

$$\frac{dn^{+\prime}}{dt} = -\frac{Ac^{+\prime}}{fN_0}\left(\frac{RT}{c^{+\prime}}\frac{dc^{+\prime}}{dx} + F\frac{dE}{dx}\right). \tag{9}$$

(Here $n^{+\prime}$ is the net number of moles of a cation species in unit time from side 1 to 2, $c^{+\prime}$ is the concentration of a cation species on side 1, and $dc^{+\prime}/dx$ the gradient of concentration of this ion across the membrane along a line normal to the surface; dE/dx is the potential gradient along the same line, A is the surface area and f the frictional resistance per unit of average velocity for the single ion along the normal line. N_0 being the Avogadro number, the expression $1/fN_0$ can be replaced by u', the absolute mobility of the cation, or its mean velocity under unit force along the line of force. It may be noted that the Fick diffusion coefficient $D = RT/fN_0 = RTu'$.)

Another treatment is possible apart from this Nernst-Planck differential equation, deriving originally from Danielli (1943) as given in Davson & Danielli's book, *The Permeability of Natural Membranes*, and by Davson (1951) in his *General Physiology*. In this there is applied to membrane kinetics the activation concepts already in use for chemical kinetics and is especially applicable to non-aqueous membranes. An important elaboration of this activated-state theory has been recently published by Zwolinski, Eyring & Reese (1949). The present treatment, which is dealt with in more detail elsewhere (Conway, 1953), follows the classical Nernst-Planck concepts.

The integration of equation (9), as Teorell (1952) points out, was given by Behn (1897) as

$$n^{+\prime} = -u' \left\{ \frac{ARTt}{\delta} \frac{(c_2 - c_1)}{(c_2\xi - c_1)} \frac{\ln c_2\xi/c_1}{\ln (c_2/c_1)} \right\} (c_2^{+\prime}\xi - c_1^{+\prime}). \tag{10}$$

(Here c_2 and c_1 are the total concentrations on each side of the membrane and δ the thickness of the membrane. A steady state is assumed.) This is the solution with a homogeneous and non-ionic membrane. Teorell (1952) has given the more complete solution, for an ionized membrane, but for the present treatment the simpler condition is sufficient.

The expression between brackets is common to the various diffusing ions, and this expression multiplied by $c_1^{+\prime}$ may be taken as the flux of the ion in one direction, and multiplied by $c_2^{+\prime}\xi$ it may be taken as the flux in the opposite direction, the ratio of the two partial fluxes being

$$-\frac{n_2^{+\prime}}{n_1^{+\prime}} = \frac{c_2^{+\prime}\xi}{c_1^{+\prime}}. \tag{11}$$

When the total electrolyte concentration on each side of the membrane approaches equality, equation (10) expressed for the partial flux from phase 1 to phase 2 changes to

$$n_1^{+\prime} = \frac{u'AtRT}{\delta} \cdot \frac{\ln \xi}{(\xi - 1)} \cdot c_1^{+\prime}, \tag{12}$$

or

$$\frac{dn_1^{+\prime}}{dt} = ART \times \frac{u'c^{+\prime}FE}{\delta} \times \frac{e^{-FE/RT}}{(1 - e^{-FE/RT})}, \tag{13}$$

which is the same as the equation of Levi & Ussing (1948) when $1/fN_0$ is substituted for u'. (A very recent article by Ussing (1952) dealing more fully with flux equations was received by the author when this manuscript was going to press.)

Considering now the net flux of the cation (here Na) across the membrane, this is also the net flux of the salt. The minimal energy expenditure in the process is made up of the reversible work done plus an irreversible loss of free energy.

The reversible work done. This may be expressed as

$$-\Delta G = 2RT\ln c_2/c_1. \tag{14}$$

(Here it is assumed that NaCl or similar salt is the only electrolyte on each side of the membrane.) The sum of the work against the potential difference for the Na and Cl across the membrane is zero.

The irreversible work. This corresponds to the loss of free energy in diffusion, which in turn may be regarded as irreversible work done against the frictional resistance to the net diffusion of molecules or ions. For a neutral solute diffusing across a membrane, this may be expressed as

$$-\Delta G = T\Delta S = \frac{nAtRTD}{\delta}(c_1 - c_2)\ln\frac{c_1}{c_2}. \tag{15}$$

This irreversible change of free energy when Na ions are actively carried is made up of such components as the diffusion to a higher chemical (but lower electrochemical) potential of the Cl ions; secondly, to the loss of free energy in the back-diffusion of Na ions which were actively carried against the electrochemical gradient; it would also be increased to some extent by appreciable water fluxes through the membrane, though this latter may here be neglected.

Loss of free energy in the diffusion of the Cl *ions*

In the present context this is considered to occur to a higher chemical potential, or $RT\ln c_2/c_1$ is a positive quantity, but to a lower electrochemical, or $(RT\ln c_2/c_1 - EF)$ is negative. This latter expression is zero when there is no net flow of the Cl ions; then

$$E = RT/F\ln c_2/c_1, \tag{16}$$

When a flow of Cl ions occurs the potential must exceed this figure. The amount may be obtained from Behn's equation above, where ξ is replaced by $1/\xi$ and u' by v', the latter being the mobility of the Cl ions.

From this equation it follows that

$$E=\frac{RT}{F}\ln\frac{c_2}{c_1}+\frac{n^-\delta\ln c_2/c_1}{FAtv'(c_2-c_1)}. \qquad (17)$$

Here n^- in a steady state is also the total NaCl transported and the amount of Na actively carried.

Transport potentials for the free ions

It is obvious that the second member on the right of equation (17) is the surplus potential carrying across Cl^- ions. One may write

$$E_t=\frac{n^-\delta\ln c_2/c_1}{FAtv'(c_2-c_1)}, \qquad (18)$$

where E_t is the transport potential. It will be seen that this is proportional to the active transport of Na ions (which in a steady state is the same as Cl^- transport) also to the thickness of the membrane, and inversely as the mobility of the Cl^- ions.

From equation (18),

$$-\Delta G_{Cl}=\frac{n^2\delta\ln c_2/c_1}{Atv'(c_2-c_1)}, \qquad (18a)$$

where n is the net transport of NaCl and $n=n^+=n^-$.

Another source of free-energy loss occurs if there is a back-duffusion of the Na^+ ions, since for any effective carriage of Na^+ ions against the electrochemical gradient, this back-diffusion represents a surplus carriage, and so much extra energy is required for a given net transport. Such energy loss is clearly the flux of Na^+ ions from 2 to 1, multiplied by $(RT\ln c_2/c_1+EF)$ or

$$-\Delta G_{Na}=\frac{Atu'(c_2-c_1)(RT\ln c_2\xi/c_1)^2}{\delta\ln(c_2/c_1)}. \qquad (19)$$

Collecting these three energy quantities one obtains

$$-\Delta G_{Total}=2nRT\ln c_2/c_1+\frac{n^2\delta\ln c_2/c_1}{Atv'(c_2-c_1)}+\frac{Atu'(c_2-c_1)(RT\ln c_2\xi/c_1)^2}{\delta\ln(c_2/c_1)}. \qquad (20)$$

Approach of the transport system to the maximum efficiency

It will be seen from equation (20) that the efficiency of the transport of NaCl increases as the mobility of the free Na^+ ions through the membrane decreases towards zero, and as the mobility of the free Cl^- ions increases. It is very probable that no appreciable amount of free energy is lost in the cyclical movements or displacements of the carrier itself in the membrane. For it we may assume no overall diffusion gradients, and that the complex as a whole is neutral.

Where the redox pump operates, efficiency will also depend on how much of the energy of the potential jump of the electrons is transferred to osmotic work on switching their passage through the membrane system. As this energy transference may reach close to 100 %, it will appear that efficiency of transport may be very appreciably increased where the anion as well as the cation is actively carried.

V. ACTIVE TRANSPORT OF Na^+ AND K^+ IONS IN YEAST: TWO DISTINCT CARRIERS INVOLVED

Active K *absorption during fermentation*

An account has been given of the active uptake of K^+ ions by fermenting yeast (Conway & O'Malley, 1946). When one part of yeast ferments with one part of 5 % glucose unbuffered and KCl is present to the extent of 100 or 200 mM/l., then K^+ ions are rapidly taken up in quantity from the suspending fluid and H^+ ions returned in practically equivalent amount, and with previous oxygenation for many hours the pH is of the order of 1·6–1·7, but if the suspending fluid is reduced to lower and lower volumes the pH approaches 1·5. In the fermenting suspension cyanide 2 or 4 mM/l. has no appreciable effect, but azide in similar strength practically abolishes the effect but increases the *amount* of alcohol produced. (It may inhibit the *rate* of alcohol production.)

Simultaneously, with the excretion of acid, there is an equivalent, or near equivalent, production of alkali inside the cells. The process is very specific for K^+ ions as against Na^+ ions, and it takes some 25 times the concentration of Na^+ as compared with K^+ to have equal uptake. Rb is taken up at about one-half the rate of K^+ ions, and both Cs and Li behave like Na.

At the end of the fermentation the K^+ ions taken up are slowly returned to the suspending fluid.

If no KCl is present in the 5 % glucose, acid is still excreted in somewhat reduced quantity, and considerably so if the yeast has been oxygenated for hours beforehand. The H^+ ions here are associated with succinate ions as succinic acid. Most of such succinate ions were already present in the yeast cells before the fermentation, but if a large amount of suspending fluid is used, and there is prolonged fermentation, new formation of succinate ions (or succinic acid within the cells) may be shown to occur throughout. (This continued formation of succinic acid is in agreement with the work of Kleinzeller, 1941.) The excretion of H^+ ions by the cells into the suspending fluid and their appearance as succinic acid is carried out by the same mechanism as when H^+ ions appear in exchange for K ions. Though the most striking lowering of the external pH is produced by the H^+ and K^+

exchange, the secretion of free succinic acid is here analogous to the secretion of hydrochloric acid by the oxyntic cells.

The redox theory. The active secretion of H^+ ions here as for the gastric oxyntic cells is explained by the redox theory (Conway & Brady, 1948; Conway, 1951, 1952). A similar theory was advanced shortly afterwards by Crane & Davies (1948). A full account is given in a recent publication (Conway, 1952); vide also the article of Davies, 1951).

Briefly, in this theory it is considered that metabolic H atoms are transferred via a flavine enzyme to a metalic respiratory enzyme in the membrane. This retains the electrons and sets free H^+ ions. The appearance of the free H^+ ions result in a potential (a transport potential as described in the previous section) which can pull across accompanying anions, or, alternatively, such are in turn actively excreted. In the case of yeast with K^+ and H^+ exchange, K^+ ions are brought across in an adsorption complex of the type discussed above, and as the electrons are passed internally K ions are liberated within the cell.

Post-fermentative permeability of yeast cells to K *ions*

If resting yeast cells are suspended and shaken anaerobically, the entrance of K^+ ions is extremely slow (Conway & Moore, 1950). After some hours labelled K^+ externally has mixed with the internal K^+ ions to only a small percentage or less.

If such yeast be shaken in air, labelled K^+ ions enter quite readily, and after 1 hr. the mixing may proceed to some 50 or 40 %.

This entrance is almost entirely abolished by cyanide, which contrasts with the effect of this inhibitor during active fermentation, but like the effect in fermentation azide almost altogether abolishes it.

It is considered that the same K-carrier is here operative as during fermentation, but cannot transfer electrons to an organic acceptor as during active fermentation, but finally to oxygen.

The active excretion of Na^+ *ions by yeast*

As mentioned above, provided Na^+ ions are in high proportion and K^+ ions absent, or in very low concentration, Na^+ can be absorbed in fermentation.

When unbuffered N/10-NaCl exists outside the cells during fermentation without any KCl very little Na ions enter; where, however, M/5-sodium citrate is present outside the cells and the yeast is suspended in 20 times its volume of this solution containing also 5 % glucose, very appreciable amounts enter after about 1 hr. fermentation. When the yeast is then washed twice with 20 times its volume of water the cells retain as much as 60 m.equiv. Na^+ ions.

The Na^+ ions may be assumed to be brought into the cells on the K-carrier, and even a small amount of K^+ ions outside will prevent the Na^+ uptake. If a series of such fermentations in M/5-sodium citrate is carried out, it is possible to exchange practically the entire amount of K ions in the yeast cell for Na ions (Conway & Moore, 1952).

The special Na-*carrier within the cells*

Once the Na ions are introduced in quantity they are actively excreted outwards by a special carrier for Na ions (Table 1), which is as much, or even more, specific for Na as the K-carrier for K ions. The existence of this special carrier is shown by the following facts.

Table 1. *Changes of* Na *and* K *content of* Na-*yeast preparations* (*given as mmol./kg. of centrifuged yeast*) *on suspension in* N/10-KCl *and in water*

Time of suspension (hr.)	In N/10-KCl		In water	
	Na change	K change	Na change	K change
0	−20·6	+25·3	−7·9	−0·3
1	−36·8	+39·8	−14·0	−0·8
3½	−40·9	+41·2	−19·8	+1·0
19	−47·8	+41·8	−23·4	+3·7

Average Na and K content of yeast at zero time:
Na content = 64·9 mmol./kg. centrifuged yeast.
K content = 84·7 mmol./kg. centrifuged yeast.

(*a*) *Specificity.* Using labelled Na^+ and K^+ in suitable experiments with the Na-yeast, it has appeared that labelled K^+ in a Na-yeast prepared as above (with about 60 m.equiv. Na and 70 m.equiv. K/l.) is not carried across the membrane in appreciable amounts over some hours whereas the Na ions are freely carried, the external fluid containing 100 mmol. NaCl and 100 mmol. KCl/l.

(*b*) *Effect of inhibitors.* Whereas azide inhibits the K-carrier system, and practically abolishes the carriage of K^+ when present in 2 mM/l. this has no effect on the Na-carrier system, as shown when Na is being excreted from a Na-yeast into water. Also if Na is being excreted at a much more rapid rate into 100 mM/l. KCl, azide reduces the Na^+ transport to the same rate as into water only.

On the other hand, cyanide (2 mM/l. strong) and anoxia strongly inhibit both the K^+ and Na^+ carriers in Na-yeast (but do not inhibit these carriers in active fermentation). Table 2 shows the effect of oxygen lack.

Table 2. *Changes of* Na *and* K *content of yeast suspended for* 90 *min.* (*at* 18° C.) *in tap water and in* M/10-KCl, *and the effect on such changes of oxygen lack.* (*The washed* Na-*yeast was suspended in* 20 *times its volume of fluid*)

Conditions	Na change mmol./kg.		K change/kg. centrifuged yeast	
	In tap water	In M/10-KCl	In tap water	In M/10-KCl
Oxygen present	−13·7	−38·0	−0·8	33·3
Oxygen absent	−3·8	−8·6	−1·7	−0·3

The Na-yeast immediately after suspending had 60 mmol. Na/kg. and 78 mmol. K/kg. of centrifuged yeast.

Interaction of the Na^+ *and* K^+ *carriers*

(*a*) If the Na-yeast is suspended in 20 times its volume of 0·1 M-NaCl no net excretion occurs over 24 hr. At the same time there is very appreciable movements of Na^+ ions across the membrane, which we may assume to be carried to and fro.

The inclusion of Na azide in the suspending fluid entirely inhibits the entrance of Na^+ ions, and as a result there is a net excretion of Na^+ ions. The interpretation here is that, without the azide, Na ions without any competition from K ions are being actively carried back into the cell by the K-carrier at the same rate as they are being brought outwards by the specific Na-carrier. Azide inhibits the K-carrier and so there is a net excretion.

(*b*) When the Na-yeast is suspended in 0·1 M-NaCl a small inclusion of KCl (0·005 M) in the fluid causes a marked excretion of Na^+ ions. The rate can be doubled or more by increasing the KCl further to about 0·025 M, but it is not further affected by increase of external KCl.

K^+ ions are now taken up in approximately equivalent exchange for the Na^+ ions excreted.

The interpretation here is not that K ions displace Na^+ ions on the Na-carrier, since the large concentration of K^+ ions inside the cells does not affect the Na^+ carriage, no appreciable amounts of K^+ ions being actively extruded; but rather that electrons on the Na-carrier are transferred to the K-carrier and K^+ ions carried inwards in consequence, the electrons being then transferred to the oxygen system and the K^+ ions liberated.

(*c*) When the Na-yeast excretes Na^+ ions into water, or into 0·1 M-NaCl plus Na azide (0·002 M) or into 0·1 M-NaCl plus 0·1 KCl plus 0·002 M-Na azide, the rate of excretion in all three cases is the same. However, when cyanide is included (2–5 mM/l.) the excretion drops towards zero.

The interpretation here is that the Na-carrier is not affected by azide, but can transfer its electrons to the oxygen system when the K-carrier is cut out but then at a much slower rate than when this is operative in the transfer of K^+ ions. Cyanide, however, blocks the path of these electrons to oxygen whether directly transferred by the Na-carrier alone or via the K-carrier.

While the 'redox-pump' theory affords an explanation for the various facts in connexion with the active transport of K ions and excretion of Na ions, the following special supporting evidence may be given.

(*a*) *The effect of water soluble redox dyes on the* K *and* H *ion exchange during fermentation*

During fermentation in the presence of N/10-KCl, with one part of centrifuged yeast to 20 volumes of 5 % glucose containing M/10-potassium succinate buffer, a steady pH value of approximately 4·5 was maintained over some hours. Conducting the fermentation anaerobically (Conway & Kernan, 1953) in a stream of nitrogen and including various redox dyes in strength M/10,000 with characteristic potentials ranging from 290 to − 160 mV, it was found that the potential is registered by a platinum electrode against the saturated calomel electrode (and calculated as against the normal hydrogen electrode) altered in correspondence with the characteristic potential of the redox dye.

Without any such redox dye the pH was about 180 mV. When it was raised beyond this value there was an increased K- and H-ion exchange. When it was reduced below this value the exchange was reduced, and could be altogether abolished at potentials at or somewhat below 100 mV.

Plotting the potentials with the dyes included against the ratio of the H-ion secretion to the control value (without any dye) gave a practically linear relation, with some scatter for different dyes.

When the H ions secretion was in this way abolished, there was almost no effect on the CO_2 or alcohol production.

(*b*) *The relation of the oxygen consumption to the* Na *output by* Na-*yeast excretion at its maximum at room temperature*

From the purely energetic standpoint when Na-yeast excretes actively into solutions containing N/10-NaCl and sufficient KCl to reach maximum output, the work done on such excretion is only a small percentage of the total energy output. When, however, the number of Na ions excreted for one molecule oxygen uptake is examined (the suspension being 1 part of Na-yeast to 100 of suspending fluid), the ratio is near to 4 for the first 50 min. In short, under such conditions, the obvious interpretation is that all or nearly all the electrons which finally reach oxygen pass through the transport system involving the Na and K carriers (Conway, Ryan & Carton, 1954).

(*c*) *The azide effect*

Azide (2 mM) inhibits the K-carrier, but not the Na-carrier. Azide in such concentration completely abolishes new formation of organically bound phosphate, even during active fermentation. From this latter action (Conway, Carton & McGovern, 1953) the transference of the oxidative energy to energy-rich phosphate bonds ceases and the practically exact agreement of the active transport of Na ions under such conditions and into water without any azide shows that the direct usage of electron energy rather than indirectly through phosphate bond energy interprets the results.

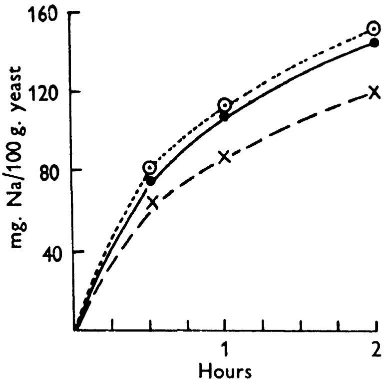


Fig. 1. Curves of extrusion of Na (as mg./kg. yeast) from a Na-yeast suspended in 20 times its volume of 0·1 KCl and 5 % alcohol (Conway & Moore, 1952) which had about half its K replaced by Na (=60 mmol./kg.). Control curve (without hormone). Curve with DOCA. Curve with 17-hydroxycorticosterone. Control curve (middle) without hormone. Curve with DOCA. Curve with 17-hydroxycorticosterone.

The effect of adrenal cortical hormones on active transport of ions in yeast

The effect on the excretion of Na from Na-yeast of various cortical hormones and other steroids included in the suspending fluid was examined (Conway & Hingerty, 1953). Ethanol to the extent of 5 % was incorporated in the fluid to assist the steroid solubility. Of the steroids examined, only DOCA, cortisone and compound F showed any effect. DOCA always definitely inhibited, cortisone to a much smaller extent, and compound F had a small but definite stimulating effect (Fig. 1). The amounts of these steroids present per litre suspending fluid were approximately 0·002, 0·0 and 0·00 mM respectively.

VI. THE ACTIVE EXCRETION OF Na IONS FROM MUSCLE

To observe this in mammalian muscle *in vivo* (Conway & Hingerty, 1946), rats were used with skeletal muscle containing relatively large amounts of Na (upwards of 50 m.equiv./kg.) as a result of feeding for a month on a K-free diet in accordance with the experiment of Heppel (1939). Such rats were then put on a diet with high K content, and the skeletal muscle actively excreted its contained Na slowly. Fig. 2 illustrates the results obtained. In about 12 days the muscle Na had decreased to normal level, and about 3 days were required for the average half-period of excretion. This may be considered the first unequivocal demonstration of active Na extrusion in quantity from muscle, and under conditions in which a considerable amount of Na had entered the muscle fibres *in vivo*.

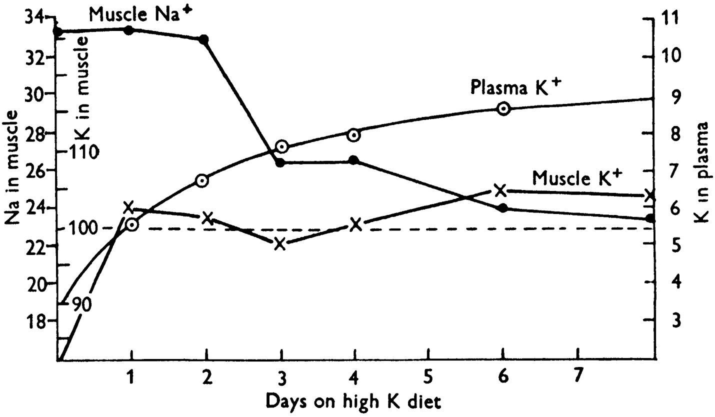


Fig. 2. Mean curve (six rats used) of Na content of skeletal muscle, after a period of K-free diet and restoration to a high K diet. The dotted line gives the normal level. Curves for plasma K and muscle K for the same rats are also given.

The question of Na *excretion from the isolated frog sartorius*

Steinbach (1940) investigated the question of the active extrusion of Na from the isolated frog sartorius when much sodium had entered the muscle fibres after 24 hr. immersion in K-free Ringer fluid. Such immersions were carried out using two sartorii from each frog. One of these was then re-immersed in Ringer fluid containing K ions (10 m.equiv./l.) and it was concluded that excretion of Na ions was demonstrated.

Such experiments have been already discussed (Conway, 1945, 1946). Steinbach has recently published experiments (1951) in which the conditions were better controlled and with 12 pairs of muscle he obtained an average of 48 ± 2·7 m.equiv./kg. after the first immersion and 39 ± 1·7 m.equiv./kg. after the second immersion or a difference of 9 ± 3·2 m.equiv./kg. While this made the excretion of Na very probable we have been unable to obtain quite the same results on repeating his procedure, due, no doubt, to some undetermined difference in technique.

Very recently (Desmedt, 1953) considerable excretion of Na has been demonstrated using single isolated sartorii immersed in Ringer fluid containing K ions. The muscles had gained much Na by previous immersion in K-free Ringer fluid. It is, however, important to note that the Ringer fluid used differed from that of Steinbach in so far as it represented the average inorganic composition of the frog's plasma (Boyle & Conway, 1941).

The localization of Na *in muscle*

It is in general assumed that when the interspace Na is subtracted from the total muscle Na, the remainder exists in the fibres and is evenly distributed through their substance. The following evidence shows that such is very probably not the case, and that most of the fibre Na is either localized in the sarcolemma, or alternatively concentrated in a small group of fibres (about 10% of the whole) with the Na content of the typical fibre, and behind the sarcolemma, only of the order of 2 or 3 mM/kg. total muscle. Such a conclusion is based on the following evidence (Conway & Carey, 1953).

(*a*) *The curve of entrance of labelled* K *into the sartorius in the cold.* When the isolated sartorius is immersed in Ringer fluid containing, say, 10 mM KCl/l., and a small amount of labelled K is introduced, this enters at first very rapidly to a level representing about 0·65 of the whole muscle. After this there occurs a very slow further entrance. This is linear up to an hour or more, and extrapolating the line to cut the ordinate gives the 0·65 fraction as given above. The half-period of this first zone is about 8 min. at or near to 0° C. (Fig. 3).

The picture is clearer if sulphate-Ringer instead of Cl is used, the Cl ions being entirely replaced by sulphate.

This first zone appeared in each of a large number of experiments conducted with all suitable precautions. It was also evident when the muscles were immersed in frog heparinized blood and a little labelled K^+ introduced, the muscles being cut at no point, and having a little of the pelvic bone attached; also their surface was not dried in any way before immersion.

It will be seen that the space of 0·65 is far higher than the interspace between the fibres, which is approximately 0·13 (Conway, Kane & O'Reilly, 1941) so that there must be some fibre region into which labelled K in the cold enters relatively very swiftly. Such a region may be the sarcolemma, or alternatively some group of fibres.

This first region of entrance, even with heparinized frog blood and muscle surface not dried was first described at the Physiological Congress in Copenhagen (1950). Very recently a similar initial swift entrance of ^{42}K into the fibres of the frog sartorius was described by Harris (1953) and attributed to entrance into injured fibres.

(*b*) *The effect of increasing the external* K *concentration on the level of this first entrance.* If K ions can enter a fibre region so quickly it seems likely that Na ions would enter it more rapidly than into the general fibre space, and in turn that such a region may well contain a high proportion of Na ions.

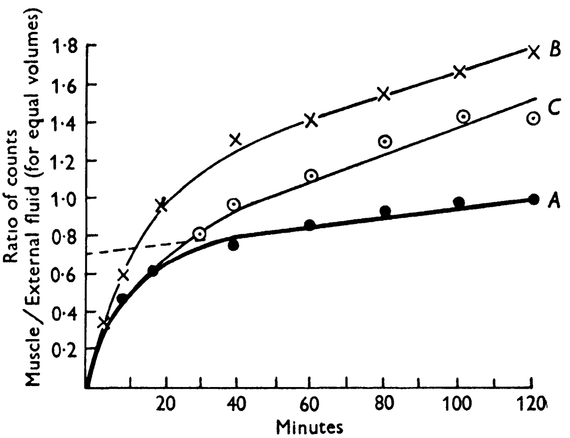


Fig. 3. Curves of labelled K entrance (given as ratios *count of* 1 *c.c. muscle*: count of 1 c.c. external fluid) into isolated sartorii at 0° C. Curve A: mean curve for six experiments with sartorii immersed in sulphate Ringer (all Cl replaced by SO_4) and K in external fluid = 10 m.equiv./l. Curve B: entrance of ^{42}K into the companion muscles used in A, immersed in chloride Ringer with 10 m.equiv. K/l. Curve C: mean entrance of ^{42}K into sartorii immersed in heparinized blood.

This may be tested by increasing the K content outside the muscle. If the region in question contains approximately the same non-diffusible anion content as the general fibres, then it should contain, if all the diffusible inorganic cations were K and Na, about 94 mM/kg. of total muscle. On increasing the K content outside up to high values the level of the first region of the curve of entrance of ^{42}K should appear to decline to about one-ninth the value when the external K was 10 m.equiv./l. The observed decline, however, is not nearly so marked, being about one-third at most. Such is readily explicable if the region in question contains a relatively large amount of Na ions with low levels of K in the external fluid.

(*c*) *The effect on the first region of immersing the muscles in* 3·2% *glucose containing* 10 *m.equiv. labelled* K-*sulphate.* The level of the first region (average of three experiments) under these conditions was found to be 0·99 as compared with 0·52 for the sulphate-Ringer (the fibre interspace being subtracted). The increased ratio is in accordance with expectation if the first region contained a high proportion of Na^+ ions, which would then exchange entirely for K^+ ions and give an increased level for the first part of the curve of entrance of ^{42}K.

A calculation of the volume of the fibre region in question may be made from such data. Thus if α be its relative volume and 94·6 m.equiv./kg. represent the total of Na plus K in the fibres (Boyle & Conway, 1941), then α is given by

$$\frac{\alpha \times 94\cdot6}{10} = 0\cdot99,$$

so that α is approximately 0·10 or 10 % of the total fibre space.

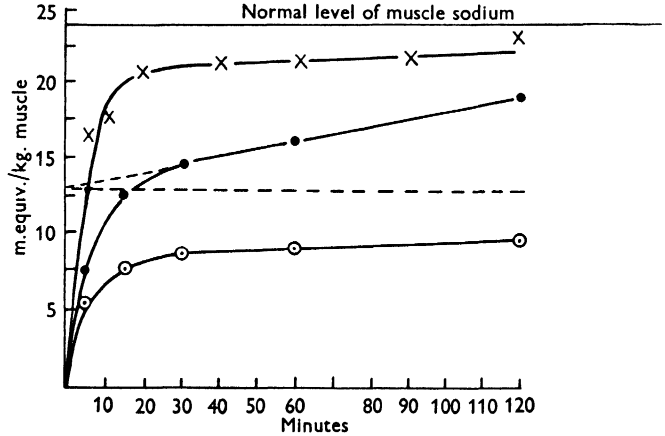


Fig. 4. Curves of Na and Cl losses from frogs' sartorii immersed in 3·2 % glucose at room temperature. Centre curve (●): Na losses into 3·2 % glucose. Lower curve (⊙): Cl losses into 3·2 % glucose. Upper curve (×): Na losses into 3·2 % glucose containing 100 m.equiv. KCl/l. Dotted line gives mean level of Cl and HCO_3 in normal muscle.

From more detailed studies it would appear that *in vivo* about 70 to 80 m.equiv./kg. of Na may be assumed to exist in this space and about 20–30 m.equiv. K. Thus it would account for about 7–8 of the 10 m.equiv. of Na in the muscle fibres. The K ratio across the membranes of such a region would be about 8–12, and could be expected to give a membrane potential of about 56 mV.

This Na region is considered from such evidence to be not only very freely permeable to K ions but also to Na ions, and that under normal conditions some active extrusion of Na allows a K increase in the region beyond the plasma level, but the major fraction of the non-diffusible anions are balanced by Na ions.

(*d*) *Sodium exit from the sartorius into 3·2 % glucose.* When sartorii are immersed in 3·2 % glucose the Cl, which is almost all in the interspace, comes out rapidly. The *interspace* Na comes out as fast as the Cl, but the *fibre* Na much more slowly (Fig. 4). Most of the fibre Na is lost when only a small fraction of the K has emerged. This may be explained by active extrusion of Na ions from the whole fibres. At the same time the difference is in large measure dependent on the localization of the fibre Na. It is apparent from the evidence given above that the fibre Na comes out for the most part from a special fibre region with a much freer K and Na permeability than the remaining region of the fibres.

If this latter conclusion be true then if 100 mmol./l. of KCl is included in the glucose, the Na^+ ions should come out far more rapidly than into the free glucose owing to free K and Na exchange. This in fact happens as shown in Fig. 4, giving the exit of fibre Na when 100 mM-KCl/l. is included in the glucose solution. Some, however, of the fibre Na remains for an indefinite period or comes out very slowly. This Na fraction may be regarded as the Na behind the sarcolemma or within the substance of the typical fibres.

Is the readily interchangeable Na *present in the sarcolemma or localized in about* 10 % *of the muscle fibres?*

The available evidence as a whole appears to favour its presence in a group of fibres rather than in the sarcolemma. The following may be noted.

(*a*) When sartorii are immersed in K-free Ringer overnight in the cold, and much Na has entered the fibres, such Na appears to come out just as readily into K_2SO_4, for example, as the normal fibre Na. Further, neither cyanide, iodoacetate nor azide when each is present as a mM/l. has any appreciable effect on the rate of emergence (Conway & Carey, 1954). Here the simpler interpretation is that individual fibres on immersion in K-free Ringer change and allow much Na to enter.

(*b*) The fact that when nearly half the muscle K has been displaced by Na, due to a K-free diet (Heppel, 1939) extending over some weeks, then ^{24}Na exchanges very rapidly with all this muscle Na. But it takes about 3 days for half of it to be extruded on return to a high K diet (Fig. 4) (Conway & Hingerty, 1946). With such diet the plasma K passes above the normal plasma value after 1 day. Exchange diffusion (Ussing, 1949) has been proposed to explain this result, but it is more simply interpreted by the alteration in permeability of a group of fibres. With this change Na ions come to move in and out freely and the fibres may then be relatively impermeable to Cl; for although much Na has entered the fibres in quantity, there is but little increase in the muscle Cl.

(*c*) At the same time Nastuk & Hodgkin (1950) have examined the potential across the membrane of a number of individual fibres and obtained an average value of about 88 mV. at 18° C. and 84 mV. at 7° C. The potentials at room temperature 'usually lie between 80 and 95 mV. Resting potentials as low as 60 mV. were occasionally observed but are regarded as due to faulty impalement.' These fibres if included 'would not have altered the average values by more than 1 or 2 mV.' If we were to take the 2 mV here and an average potential for the accepted fibres as 84, then 8 % of the whole fibres giving 60 mV. would only alter the 84 to 82 mV., and 8 % is close to the 10 % considered above and which can only be considered a very approximate figure.

Table 3. *Relative net entrance rates of ions into muscles compared with the relative theoretical diffusion constants through water*

Cation series		'*D*' for single ions, with K value = 100	
KCl	100	K	100
RbCl	38	Rb	103
CsCl	8	Cs	104
NaCl	0	Na	67
LiCl	0	Li	52
$CaCl_2$	0	Ca	40
$MgCl_2$	0	Mg	35
Anion series			
KCl	100	Cl	100
KBr	63	Br	105
KNO_3	17	NO_3	96
K phosphate	4	H_2PO_4	50
$KOOC.CH_3$	3	HPO_4	39
$KHCO_3$	1	CH_3COO	54
K_2SO_4	0	SO_4	53

The values of *D* were determined from the formula RT/zF^2. The entrance rate of K phosphate is for nearly equal mixtures of K_2HPO_4 and KH_2PO_4. (Table from Conway, 1947; entrance rates of salts from Conway & Moore, 1946.)

At the same time no very definite conclusion can be drawn as to the region of localization. It may also be pointed out that for experiments where exact figures are advanced for the Na ion rates across the muscle-fibre membrane (e.g. Harris & Burn, 1949), the result is complicated by the localization of the fibre Na in two regions, from one of which the Na will move rapidly, and from the other very slowly. An incorrect comparison could thus be drawn between the rate of passage of free K and Na ions across the typical muscle-fibre membrane.

Passage of K *ions across muscle and nerve-fibre membranes*

Whereas in yeast the passage of K ions into the cell is dependent on an active transport mechanism the passive entrance of the free ions being relatively very slow, the entrance of K and Cl ions into muscle was shown to be a passive process (Boyle & Conway, 1941), and similarly for liver cells (Conway, 1944) and the cells of the proximal convoluted tubules of the frog's kidney (Conway, FitzGerald & MacDougald, 1946). Shanes (1946) showed such permeability to apply also to nerve fibres, working with spider-crab nerves; and this was also found applicable to nerve fibres by Hodgkin (1947) and by Hodgkin & Huxley (1947).

Immersion of frogs' sartorii in the cold in Ringer solution containing various amounts of KCl and NaCl produced equilibrium values of K, Cl, water content and of resting potentials which were predictable from a consideration of simultaneous Donnan, electrical and osmotic equilibria.

It appeared that for membranes of the soft tissues of the body in general a certain type of equilibrium applied which may be described as follows:

(*a*) Below a certain size level solute particles will penetrate the cell membrane freely, independently of their lipoid solubility.

(*b*) Small cations and anions can in general both pass the cell membrane with varying degrees of freedom.

(*c*) The critical size in general for rapid passage of cations is at the K level (hydrated ion) or between it and that of the Na ion, and the critical size for the anions is at or near the dimensions of the Cl anion (Table 4). In short, for free entrance of cations or anions, it is approximately 8 Å. diameter. Thus, while K, Rb and Cs ions can enter the cell at appreciable rates over short periods, Na and Li ions are virtually excluded; and while Cl, Br and NO_3 ions enter freely, HCO_3 and CH_3COO ions diffuse very slowly and SO_4 ions are practically excluded. That the ion size is not the sole determinant of the entrance rate is shown by the fact that with muscle Cs enters much less rapidly than K. (The same would appear also for nerve, though the difference is less marked (Hodgkin, 1947).

Table 5 shows the relative rate of ions into muscle fibres with the relative theoretical diffusion constants through water.

Table 4. *Ion sizes and hydration*

	Ionic radii		Hydration (mol. H_2O)
	Hydrated	Non-hydrated	
Rb	3·6	0·49	0·5
Cs	3·6	1·65	0·2
K	3·8	1·33	3·8
Na	5·6	0·98	8·0
Li	7·3	0·78	12·6
Ba	8·8	1·43	13·5
Ca	9·6	1·06	17·6
Sr	9·6	1·27	14·6
Mg	10·8	0·78	22·2

Table from Conway (1947); data compiled from various sources. Ionic radii in Ångstroms.

From various reviews by other workers, e.g. Hodgkin (1951), Keynes (1951), Ussing (1949) and Katz (1952), it will be seen that the general nature of the above views have been accepted.

With respect to muscle-fibre sodium, Krogh (1946), relying on the isotope studies of Heppel and others, concluded that Na ions entered the muscle fibre as fast as, if not faster than, K ions. This was shown to be erroneous from energy considerations (Conway, 1946), and from the energy requirements alone Na could enter only at a fraction of the rate of K ions, there being a constant active extrusion of the entering Na in the steady state.

Maizels (1951) has represented us in this connexion as holding that Na could not be actively excreted because of energy requirements. The point made against Krogh's views was, however, that Na could not be actively excreted (and a steady state reached) if Na ions enter *as fast* as K ions per unit concentration. From the energy calculations they could only enter much slower.

If one judges the true rate of passage of Na ions and their active extrusion from the muscle fibres *in vivo* then the rat experiments (Conway & Hingerty, 1946) commented upon above show a half-period of some days for extrusion of Na which had entered muscle in quantity. Hodgkin & Huxley (1947) find that free Na ions move across the fibre membrane of the squid axon at only about one-fortieth the rate of K ions.

For isolated sartorii muscle Harris & Burn, using isotopes, give the rate for K ions as about seven times that of Na ions, but as discussed above the result for Na ions will give in effect only the rate from localized regions of Na accumulation. The true rate for the typical muscle fibre is probably much slower than that given by Harris and Burn (1949).

VII. THE ACTIVE TRANSPORT OF Na IONS ACROSS THE FROG'S SKIN

The study of the electrical phenomena of the frog's skin and its active transport of ions has had a long history proceeding from Du Bois Reymond (1848), through Galeotti (1904) and many later observers. In recent years much valuable work has been done by Ussing and co-workers (*vide* Ussing, 1949, 1952). In a recent important paper by Linderholm (1952) from Teorell's laboratory, a theoretical introduction of interest is given, also a historical review. Linderholm confirms the findings of Ussing & Zerahn (1951) and adds much new material. He interprets his results with respect to the active transport of Na ions in terms of the 'redox pump' (Conway, 1951).

When a counter potential is applied across the mounted frog skin to reduce the potential difference across the skin to zero a current flows in the controlling circuit which is entirely due to actively carried Na ions (as shown by Ussing & Zerahn (1951) and confirmed by Linderholm (1952)), no net exchange of Cl ions occurring.

The oxygen consumed and Na ions transferred in these short-circuited skins is 4·8 μl. O_2 cm.$^{-2}$ hr.$^{-1}$, and 238 $\mu\mu$F. cm.$^{-2}$ sec.$^{-1}$ respectively. The ratio of Na ions carried to oxygen molecules consumed is therefore 3·4. The theoretical ratio, if all the electrons received by the oxygen were diverted to Na carriage, is 4·0. The Na ions would appear to be carried by the epithelial cells of the skin which have a high oxygen consumption (Erdman & Schmerl, 1926). Some oxygen is no doubt consumed by other cells of the skin, but it is quite probable that the epithelial consumption accounts for nearly the full amount.

Linderholm also discusses other conditions, in which the ratio would appear also in harmony with the theory. Such ratios may be compared with those found in the active extrusion of Na by yeast dealt with above. He points out too that the theory of the 'redox pump' readily explains the current produced by the skin, and the dependence of the electrical potential on the oxygen supply as well as the inhibition of the potential by several respiratory inhibitors.

VIII. SOME RECENT VIEWS CONCERNING THE NATURE OF GASTRIC ACID SECRETIONS

Work on this question up to 1950 was reviewed by the author (Conway, 1952), and while this was in proof a review on the same subject by Davies (1951) appeared.

Nielsen & Rosenberg (1951) have advanced a theory, involving the transfer of energy by ATP in the formation of a monometaphosphate ester of a sterol, and the change of this by enzyme action into a derivative of orthophosphoric acid with H-ion increase in the membrane (which is anion- but not cation-permeable). Cl ions are exchanged for the organic phosphate ions across the membrane which in turn are split by a phosphatase, and the original sterol or sterol-like substance reformed. Discussion must here be limited to pointing out that the existence of the required monometaphosphate ester and the special enzyme have not been demonstrated. Also the theory was advanced chiefly to account for the very high HCl/O_2 ratios recorded by Davies which have been recently criticized by Davenport (Davenport, 1952; Davenport & Chavré, 1952).

Another kind of theory has been advanced by Hogben (1951). In this the H ions of the gastric juice derive from the reaction

$$H_2CO_3 \rightleftharpoons H^+ + HCO^-$$

in the canaliculus, HCO_3^- being exchanged for Cl^- ions actively transported across the membrane, leaving H^+ and Cl^- ions. The assumptions in this theory are too considerable to make it plausible. Thus it is assumed that Cl^- on a carrier is displaced by HCO_3^- in a concentration of the order of 10^{-9} M, or that the carrier is about 10 million times more specific for HCO_3 than Cl ions in the membrane or on the edge of canaliculus, while inside the substance of the cell the carrier is relatively *specific for* Cl *ions*, since the efficiency of the whole process depends on this specificity. Further it is assumed that the moment HCO_3^- is in this way attached to the carrier its energy level is raised by about 10,000 cal. of free energy per ion equivalent, and no indication is made as to how this happens. It is also assumed that a back-flow of HCO_3^- into the canaliculus occurs, or that the membrane is permeable to free HCO_3^- ions, in which case a very high diffusion potential (about 350–400 mV.) could be expected.

Also, the frog's mucosa contains at least three different kinds of cell lining the boundary between the serosal and mucosal sides, and to produce an overall electrochemical potential difference of zero across the membrane does not necessarily imply that this holds for the oxyntic cells; and the evidence for the active carriage of Cl ions (which has never otherwise been shown for animal tissue cells) should be examined in the light of this objection. Finally, it may be remarked that carrier transport of Cl ions would be quite in harmony with the 'redox theory' (vide Conway, 1952), in which it is not assumed that H ions are transported but rather metabolic hydrogens. These are transferred in a similar way to normal metabolism except that a localization of certain phases occurs in the membrane.

SUMMARY

1. Mechanisms for the immediate energy source in active transport, chiefly the 'redox pump', have been discussed.

2. The energy required in active transport, involving reversible and irreversible energy changes, have been expressed in equational form.

3. The significance of 'transport potentials' has been examined.

4. Recent developments in the nature of Na- and K-active transport in yeast have been described. There are two distinct carriers, one specific for Na, which removes Na from the cell, and one specific for K, which introduces K into the cell. Azide (2 mM/l.) does not inhibit the Na-carrier but cyanide and oxygen lack practically entirely inhibit both. The cyanide inhibition does not occur if the cells are rapidly fermenting glucose.

5. DOCA (and to a lesser extent cortisone) in 0·025 mM/l. concentration markedly inhibits the extrusion of Na ions from Na-yeast immersed in a

KCl solution, but it does so indirectly by its effect on the K-carrier. Compound F stimulates the Na excretion or the K uptake.

6. Evidence is advanced for the view that the fibre Na in skeletal muscle is either localized in the sarcolemma, or exists in a special group of fibres.

7. Criticisms have been made of recent theories of the nature of HCl secretion.

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CATION ABSORPTION BY NON-GROWING PLANT CELLS

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I. INTRODUCTION

The uptake of inorganic solutes by plants clearly involves a complex of interrelated processes, any one of which may, in suitable circumstances, limit the overall absorptive capacity of the organism. Amongst these controlling factors are the rate of utilization and the translocation of ions from one part of the plant to another. Both of them are intimately related to growth, and the influence which growth exerts on the course of mineral-salts absorption may be attributed at least in part to these associated processes. Helder (1951) has shown that the uptake of nitrate by maize plants is dependent upon its incorporation in the organic constituents of the cells. Numerous researches with animal tissues (Kamen & Spiegelman, 1948; Sacks, 1948) have indicated that the mechanism of phosphate absorption cannot be divorced from the functional importance of phosphorus in metabolism, and the same is probably also true for plants.

In order to distinguish utilization from a more fundamental absorption mechanism, much attention has been paid to the uptake of such ions as potassium and chloride, which are not appreciably metabolized, but accumulate within plant cells. Rubidium and bromide ions have often been favoured as indicators (Steward, Prevot & Harrison, 1942), since they do not usually occur in plants at all. There is no evidence that the absorption mechanism for these relatively metabolically inert ions is in any way fundamentally different from that which is involved in the case of nitrate or phosphate. On the contrary, it is probable that the uptake of inorganic solutes always involves a combination with organic cell constituents. But whereas with nitrate, for example, these become immediately concerned in metabolic processes leading to the synthesis of protein, in the case of accumulated ions, the complex is subsequently broken down to release free ions into the cell vacuoles. In the present study, attention is confined to the absorption of two cations, potassium and sodium, which accumulate readily within the tissues under investigation.

In order to reduce the effect, on absorption, of the transport of materials away from the absorbing region, frequent attempts have been made to examine ion-uptake, using less complex systems than those which are presented by the intact angiosperm. Some investigations have been made upon the coenocytic algae such as *Valonia*, *Halicystis* and *Nitella* spp., where the problem of translocation is not involved (Hoagland, Davies & Hibbard, 1928; Brooks, 1937; Jacques, 1938). Mainly because of the difficulty of culturing them in the laboratory, these algae, however, are inconvenient material for most absorption studies, and many research workers have favoured the use of excised roots as experimental objects (Lundegårdh & Burström, 1933; Hoagland & Broyer, 1936; Humphries, 1950). Excised roots are easy to grow in culture under controlled conditions; they are the natural absorbing organ of the plant, and when isolated they absorb ions rapidly without the modifying influence of transport into the shoot.

That the movement of ions away from the region of absorption may still exert a complicating effect with this material is, however, indicated by the suggestion of Lundegårdh (1949) that mineral salts may be moved across the cortex of excised wheat roots into the conducting elements of the stele, and from there, through the cut surface, back into the culture medium. Investigations with excised roots are further complicated by the inevitable presence in the organ of many different types of cell with diverse absorptive capacities. Prevot & Steward (1936) found that there is a pronounced longitudinal gradient of accumulation along intact roots, and differences of this kind make it difficult to interpret results based on a net uptake of solutes at the cell level. This difficulty is enhanced when the relationship between respiration and absorption is being studied, since the respiratory activity of different cells in such a complex is also not the same.

Another important type of material which has been extensively used in ion-absorption studies consists of tissue slices of various storage organs (Nathanson, 1904; Stiles & Jorgensen, 1915; Steward, 1937; Robertson, 1941). An advantage of these objects is the greater uniformity of cells comprising the tissue, but the intensive investigations of Steward and his collaborators, with disks of potato-tuber tissue, have shown that even this material is not without certain complicating features. Freshly cut slices of the tissue are not immediately capable of accumulating ions metabolically, but a capacity to do so develops when the material is suspended for several days in an aerated solution of mineral salts. During this treatment there is an increase in the rate of respiration, protein synthesis begins, and the cells at the surface of the block may show a tendency to divide, forming a layer of callus (Steward, Berry, Preston & Ramamurti, 1943). A study of the absorption of ions by disks of different thickness led to the conclusion that only the cells at the surface of the slices were involved in metabolic absorption. Further, Steward & Preston (1940, 1941) demonstrated a close relationship between the rates of ion absorption and protein synthesis under various conditions.

It would appear, however, that a general application of the hypothesis, that salt absorption and protein synthesis are directly related, cannot be upheld. Ulrich (1941) has observed that there were no significant changes in the amounts of amino- or amido-nitrogen during the accumulation of ions by excised barley roots. Furthermore, it is by no means certain that, in normal circumstances, when protein synthesis is proceeding in roots, the regions of most active absorption and synthesis exactly coincide (Kramer & Wiebe, 1952).

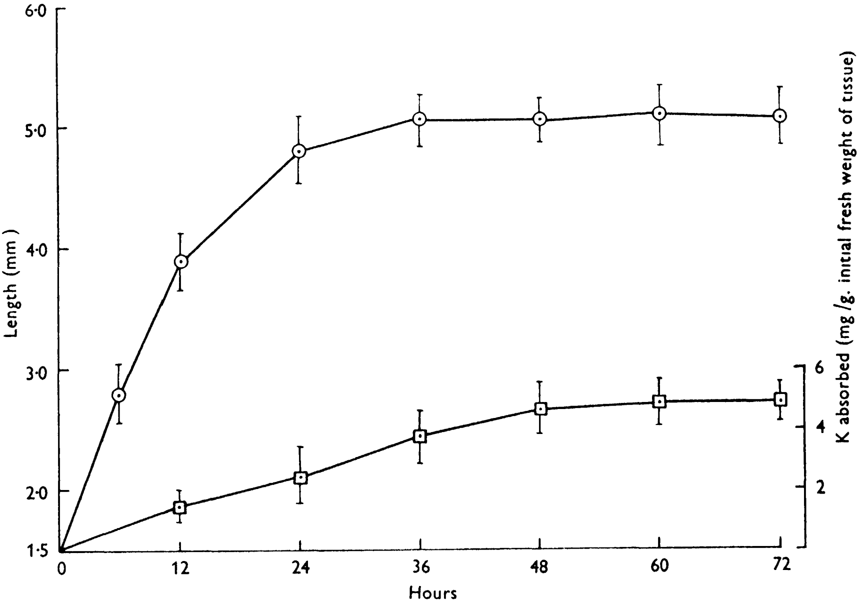

Fig. 1. Increase in length (—⊙—) and K absorbed (—⊡—) by maize-root sections placed in 2 % sucrose + 0·01 M-KCl at 25° C. during 72 hr.

Nevertheless, it is evident that the growth of cells is of profound significance in relation to absorption, and it is therefore of interest to examine the extent to which other features associated with growing cells, such as increasing surface area or volume, are important controlling factors in situations where protein synthesis does not appear to be intimately involved. Jacques (1939) concluded that there is little connexion between surface area and the rate of uptake of ions by *Halicystis*, but Burström (1942), on the other hand, has shown that there is a close similarity between the changes in cell length and the amounts of osmotically active materials in actively growing epidermal cells of wheat roots.

I (Sutcliffe, 1952*b*) studied the course of potassium uptake by extending root segments of maize, and found that the rate of accumulation remained constant during the growth phase, whilst the surface area of the tissue increased by more than 200% (Fig. 1). A similar observation has been made by Brown & Cartwright (1953). There is no significant increase in the amount of protein during the growth of these fragments, and it may be concluded that the rate of absorption of ions in this case is related to the bulk of the protoplasm, rather than to the surface which it presents to the external medium. After growth of the segments had ceased, accumulation gradually stopped, and here the finite volume of the mature tissue may be the limiting factor.

II. THE DEVELOPMENT OF AN ABSORPTIVE CAPACITY IN NON-GROWING CELLS OF STORAGE TISSUE

The study of solute accumulation by actively growing cells, even in the simplest experimental situation, presents such a complicated picture that it seemed advisable to get more information about the course of absorption with non-growing cells. Successful investigations of this kind have already been made with mature animal cells, such as erythrocytes (Solomon, 1952). Cells of red beet root proved to be a satisfactory plant material for this study. The tissue was cut into small disks, 0·75 cm. in diameter, and 0·5 or 0·75 mm. in thickness. After cutting, the disks were observed to swell in water during 24–48 hr., by about 10% of their original volume, and then there was no significant change for many days. Cell counts showed that there were no cell divisions during this time, and the amount of protein synthesis was small. On these grounds it was concluded that the material consisted of mature cells.

Experiments showed that with disks that were thinner than 1 mm. absorption was directly proportional to the total number of cells present, and hence it can be justifiably claimed that all the cells of the tissue, irrespective of their position in the block, are involved in accumulation. By using small disks, it is possible to select uniform groups of cells in the material, avoiding the anomalous phloem rings occurring in beet, and data thus obtained may be interpreted at the level of individual cells.

Beet tissue resembles that of potato, inasmuch as the absorption of ions by freshly cut disks is restricted, and metabolic accumulation may be stimulated by washing the material in aerated distilled water or a mineral salts solution (Fig. 2). During this treatment there is a gradual increase in the rate of respiration (Bennet-Clark & Bexon, 1943; Stiles & Dent, 1947) resembling the changes that were observed by Steward & Preston (1940) with potato. In the case of beet, however, it is likely that the changing rate of respiration is associated with increasing protoplasmic activity, involving little overall synthesis of protein.

Lundegårdh (1940) and Robertson & Turner (1945) have claimed that, whilst there may be no quantitative relationship between the total respiration of a tissue and ion absorption, accumulation of solutes is closely related to a cyanide-sensitive respiratory component, which is stimulated by the presence of mineral salts. An attempt was therefore made to observe changes in this aspect of respiration during the development of absorptive capacity in beet disks. It was found that although a solution of 0·02 M-KCl had no influence on the rate of respiration of freshly cut tissue, oxygen absorption was stimulated by salt after the material had been washed in distilled water for several days. The level of the salt-induced respiration reached a maximum after about 7 days in water at 25° C., which was also the time required for the development of maximum absorptive capacity under the same conditions. Finally, it was shown that the salt-stimulated component of respiration was cyanide-sensitive.

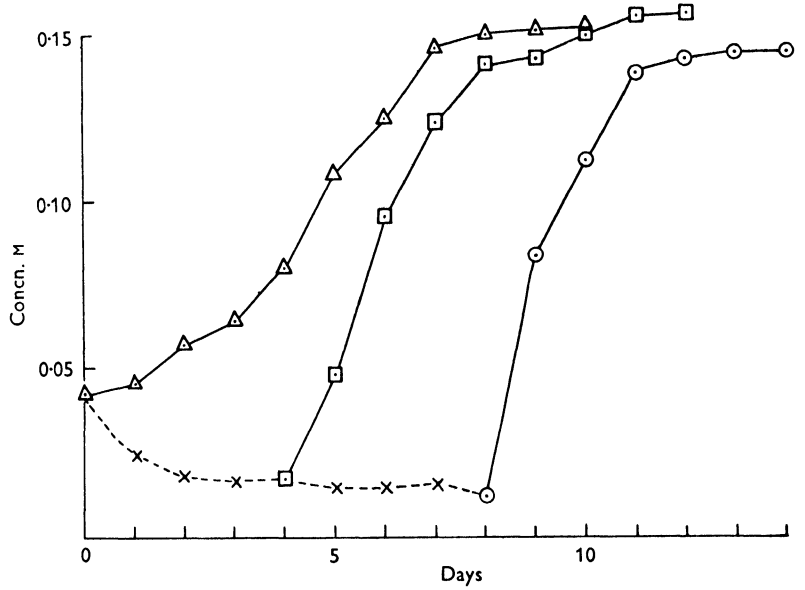

Fig. 2. Internal K concentration of beet disks during aeration in distilled water (- - × - -) and c·02 M-KCl (——), after a preliminary period in distilled water of a few hours (△), 4 days (□) and 8 days (⊙) at 25° C.

These observations support the contention of Lundegårdh that ion absorption and cytochrome-mediated respiration are closely related. But the evidence is not conclusive that cytochrome acts directly as the carrier for anions, in the way that is suggested by this worker. It is possible that many, if not all, of the energy-requiring processes in most plant cells are linked to the cytochrome terminal oxidase system, and salt absorption may not be especially favoured in this respect (Lemberg & Legge, 1949). Studies of the effect of various respiratory inhibitors on growth, for example, by Hackett & Schneiderman (1952), have shown the importance of cytochrome-mediated respiration in this connexion.

The intensification of metabolic activity during the washing of tissue slices may in part be attributed to increased oxygen tension, lowered carbon dioxide concentration, and the higher temperature to which the cells are exposed after cutting. It is likely that, through the influence of these factors, new active elements of the respiration machinery are synthesized, and cytochrome components are probably particularly involved. Parallel changes in the activity of cytochrome upon aeration of yeast cells were observed by Chin (1950).

Another possible cause of the increased rate of respiration of washed tissue is the removal of a metabolic inhibitor from the material. Skelding & Rees (1952) have demonstrated the presence of an inhibitor of ion absorption in an extract from freshly cut slices of beet, and they claim that this is gradually removed from the cells during washing, partly by diffusion into the bathing medium, and partly by metabolic degradation. Skelding & Rees found that the inhibitor does not affect respiration, and they have postulated therefore that its influence on ion absorption may be exerted through a physical effect on the permeability of the cell protoplasts.

Using the same technique as that of Skelding & Rees, I obtained a diffusate from beet tissue which inhibited the absorption of potassium by washed disks, but, at the same time, strongly stimulated respiration. A part of the respiratory effect may be attributed to the presence of various organic acids in the extract. Bennet-Clark & Bexon (1943) caused large stimulations of respiration by applying expressed sap from beet tissue to the outside of intact cells, and they concluded that part of the effect was due to the presence of malic and citric acids. It is unlikely, however, that either of these substances can be identified as the absorption inhibitor, since Machlis (1944) has shown that the influence of organic acids on ion accumulation by barley roots is rather one of stimulation than the reverse.

Until it is possible to separate the inhibitor from other metabolically active substances in the beet extract, it cannot be established with certainty whether, or not, it also affects respiration. When this point becomes clear it will be possible to understand, perhaps, how the inhibitor functions in preventing accumulation, and its relationship to the increased metabolic activity following the washing of disks.

The diffusion of various substances from freshly cut tissue which stimulate respiration may account for the phenomenon of 'wound respiration' which has often been observed with storage tissues, as well as with other plant materials (Steward, 1933; Robertson, Turner & Wilkins, 1947). The absence of this temporary stimulation of respiration, imposed on the gradual increase in metabolic activity, in our experiments, in those of Bennet-Clark & Bexon, and of Steward & Preston (1940) may perhaps be related to a more rigorous washing procedure in these cases, which prevented the accumulation of respiratory active substances in the medium.

III. THE EFFECTS OF PRETREATMENT ON THE ADSORPTIVE CAPACITY OF DISKS

There is now a considerable amount of evidence that an adsorptive phase is closely associated with the overall accumulation mechanism for cations (Lundegårdh, 1946; Overstreet & Jacobson, 1946). An investigation was therefore made of the effect of washing on the adsorptive capacity of beet disks, in an attempt to correlate it with the metabolic changes and the increasing rate of accumulation that have been described above. The adsorptive capacity of the material was determined by taking batches of disks which had been washed in distilled water for various periods of time, and placing them in a solution of 0·02M-KCl for 4 hr. at 7° C. At the end of the experimental period, an analysis of the medium was made, and the non-metabolic uptake of potassium was determined. The data obtained (column A of Table 1) show that, when the disks were transferred, immediately after cutting, to the experimental conditions, there was a loss of ions from the tissue to the external medium. After washing the material for 2 days in aerated distilled water, however, there was an immediate uptake of salt on transference to KCl. The amount of this non-metabolic absorption was increased with a longer period of pretreatment up to 6–8 days.

A comparison of the amounts of potassium adsorbed and the amounts lost from the tissue during washing (column C of Table 1) shows that, although a part of the adsorbed potassium may be replacing that which is leached from the cells, this cannot be the only factor involved since the adsorptive capacity of the disks continues to increase after the material has been washed in water for several days, when the total potassium content is no longer decreasing. Ions which are taken up by the disks may be located in intercellular spaces, cell walls, protoplasts, or vacuoles. Since the volume of the intercellular spaces and cell walls probably does not increase after about 2 days, it may be concluded that the increased adsorptive capacity of the cells is associated with an increased affinity of the protoplasm for ions, or else to a greater fixation of potassium in the vacuoles. It is likely that non-metabolic adsorption occurs in the protoplasts rather than in cell vacuoles, since Brooks (1937) and Hoagland & Broyer (1942) with *Nitella* have observed that the movement of ions into the protoplasm is rapid, whilst subsequent entry into the vacuole takes place slowly and, under anaerobic conditions, perhaps not at all.

Table 1. *Amounts of potassium adsorbed in 4 hr. at 7° C.* (A), *amounts of potassium subsequently exchanged in 6 hr. at 7° C.* (B), *and the total amounts of potassium in beet tissue.* (C), *after washing in aerated distilled water at 25° C. for various periods of time*

Days of washing	Amounts of K (μg./g. fresh wt.)		
	A	B	C
0	−50 ± 20	645 ± 43	2315 ± 175
2	185 ± 38	587 ± 38	1950 ± 163
4	415 ± 39	455 ± 29	1895 ± 122
6	699 ± 51	405 ± 32	1905 ± 148
8	731 ± 46	413 ± 30	1875 ± 143

Further evidence in this connexion has been obtained by studying the non-metabolic exchange of potassium by beet disks with ^{42}K. The tissue, after being washed for various lengths of time, was allowed to adsorb potassium for 4 hr. at 7° C. as described above. It was then transferred to a 0·02M-KCl solution containing the isotope for 6 hr. at 7° C., after which the radioactivity of the medium was determined, and the amount of potassium exchanged with the material was calculated. The results of this experiment, shown in column B of Table 1, indicate that the amount of potassium exchangeable in 6 hr. under the conditions of the investigation decreased during the washing of the tissue. After about 4 days of pretreatment on transferring the material to KCl only a part of the potassium adsorbed, exchanged readily, and about 300μg. out of more than 700μg. adsorbed per g. fresh weight of well-washed tissue, did not exchange.

The exchange data indicate that the adsorbed ions exist in at least two different states outside the vacuoles. Those which are easily exchanged may be present in the intercellular spaces, cell walls, or loosely bound in the protoplasm, whilst the rest are probably located entirely in the protoplasts. Jacobson & Overstreet (1947), from their study of isotopic exchange in roots, were led to the conclusion that cations may be bound into plant cells with varying degrees of non-exchangeability. They believe that the binding of cations in the form of chelated complexes with proteins, amino-acids, or organic acids, may be of particular importance in relation to accumulation. Ion bonds formed in these combinations are relatively strong, and ions so held are exchanged only with difficulty. The adsorbed potassium may become increasingly bound in such forms following the pretreatment of disks, and if these combinations are important in relation to the accumulation mechanism as a whole, then the increased capacity of the tissue to absorb after washing may be accounted for.

The relatively large amount of the adsorbed potassium, which becomes bound in the non-exchangeable form in washed tissue, suggests that the adsorptive centres involved are located throughout the protoplasm, rather than only at the outer surface of the cells. Suggestions that protoplasts as a whole contain negatively charged immobile anions, to which cations may be fixed, have already been made by Blinks (1940) and by Robertson (1951). It is not possible to make a calculation of the 'apparent free-space' into which ions move non-metabolically from the present experiments as, for example, Hope & Stevens (1952) have done for bean roots, since the concentration of ions which is attained in this region cannot be determined. Nevertheless, it seems unlikely that the surface of the protoplast is the sole region of adsorption as Lundegårdh (1940) suggested.

It appears to be more likely from the present data that there is a barrier to the free diffusion of ions, and to exchange processes in the vicinity of the tonoplast, separating the protoplast from the vacuole of a plant cell. Arisz (1945) has concluded, from his studies of ion absorption by leaves, that the tonoplast is the region of a cell across which active accumulation occurs. There is some evidence from isotopic investigations with algal cells (Hoagland, 1944) that the concentration of ions in the vacuoles may be greater than in the protoplasm, and this again supports the idea that ions do not move readily by diffusion across tonoplasts. This hypothesis is also in agreement with the observation of Bennet-Clark & Bexon (1943) that metabolically important substances, such as organic acids, may be relatively inactive when they are confined to cell vacuoles.

During the washing of freshly cut disks there is clearly an activation of the metabolic mechanism by means of which ions are able to traverse this physical barrier against an activity gradient. This is associated with an increased capacity of the cells to adsorb ions strongly, and it may be postulated that the formation of complexes with particular cell constituents forms an essential intermediate link in the movement of solutes from an external medium into vacuoles. Metabolic energy is probably involved in the preliminary synthesis of the carrier molecules which occurs during the washing treatment. In this way the importance of aeration and temperature in determining the rate at which the absorptive capacity of the tissue is developed, and the effect of respiratory inhibitors in retarding this process can be explained.

The fact that potassium appears to combine with the carrier at low temperature in the present experiments indicates that metabolic energy is not involved at this stage. But it may be required during a series of reactions leading to the subsequent breakdown of the complex, and the release of free ions into the vacuoles. The spatial separation of ion adsorption on to the carrier, and their release, which is responsible for actual transport may be accomplished by an aggregation of the carrier substances in microscopic particles, such as mitochondria, which undergo random movements in the protoplasm (Robertson, 1951), from a region where the ions are taken up to one where the breakdown of the complex occurs; or the contraction of protein molecules, as has been suggested by Goldacre (1952), may be involved.

IV. THE COURSE OF ION ABSORPTION WITH WELL-WASHED TISSUE

When beet disks have been washed in aerated distilled water at 25° C. for about 7 days, a maximum absorptive capacity is attained. If, at the end of this time, the material is transferred to a solution of mineral salts, absorption occurs at a rate which is dependent upon various external factors, such as the nature of the ions involved, and their concentration; the presence of other ions, or various substances which may influence metabolism; temperature, and aeration. The effects of these factors on absorption are mostly well understood, and will not be further discussed here.

There are, however, in addition, various internal influences which are effective in controlling the rate of absorption. One of these is clearly the capacity of the accumulation machinery, already considered above as a limiting factor during washing, and another is the internal concentration of ions. A reduction in the rate of uptake of solutes, when the mineral-salts content of the material is high, has been observed by a number of workers, including Hoagland & Broyer (1936) with barley roots, Jacques (1938) with *Valonia*, and Alberda (1948) working with whole maize plants. The same effect with beet disks is shown in Fig. 2.

Although the phenomenon is well established, the mechanism of it is not yet clearly understood. Broyer (1951) wrote: 'If roots (however) have accumulated inorganic solutes in the past, under favourable environmental conditions, approaching upper limits imposed by their hereditary potentialities, they may be restricted under such circumstances from further accumulation. Such high-salt roots are close to their dynamic equilibrium relative to inorganic solutes.'

The establishment of an equilibrium of the type visualized by Broyer is one of the characteristics of ion accumulation by non-growing cells.

Growing tissues are capable of absorbing ions indefinitely, and this may be due in part to the increasing volume of the material which prevents the internal concentration from attaining its limiting value. Utilization and translocation are also obviously important, in this connexion, in situations where they are operative; and protein synthesis, in so far as it results in the production of new units of the absorption machinery, must also have a modifying influence.

In studying the effect of internal concentration on the uptake of ions by non-growing cells, various alternative hypotheses may be considered. It is possible, for example, that the net accumulation of solutes is the resultant of an absorption and a leakage process, which are mutually opposed (Krogh, 1946). Assuming that uptake is metabolically controlled, and that leakage occurs passively by diffusion along a concentration gradient, as more ions are accumulated the rate of outward movement will increase. If uptake remains constant, then the rate of accumulation will be reduced, and an equilibrium will be established when the opposing processes are equal. Evidence that the absorption mechanism itself may be relatively unaffected by the increasing ion content of the material is provided by the observation (Sutcliffe, 1952*a*) that the level of the cyanide-sensitive respiration in a cell which has become saturated with potassium is as high as that of one which is still absorbing ions rapidly. If the energy of salt respiration is, in fact, involved in accumulation, then it is clear that this energy is still available after net uptake has ceased, and it may be postulated that ions are still moving into the tissue, but are being balanced by a contrary movement in the opposite direction.

There are, however, a number of reasons why this attractive hypothesis must be discarded in the present case. It has been observed, for example, that variation of the temperature between 15 and 30° C. does not alter the equilibrium position with beet disks, although it does of course profoundly affect the time which is required for the establishment of it. Even though Danielli (1952) has explained that diffusive processes through cell membranes may have a high Q_{10}, it seems unlikely that a metabolic absorptive process and a passive leakage will be influenced simultaneously to the same extent by temperature. Yet this must be the situation if the equilibrium is established in the way outlined above.

Moreover, if there is an appreciable passive leakage of inorganic ions from intact cells and tissues, it ought to be possible to detect this by observing changes in the composition of the external medium, when the material is placed under conditions which prevent metabolic reabsorption. Actually, Hoagland & Broyer (1942) found that the rate of outward diffusion of ions from excised barley roots, when oxygen is withheld, was very low. A similar observation was made with beet disks when the accumulation mechanism was inhibited by KCN (Sutcliffe, 1952*a*).

Although the evidence appears to be conclusive that there is no significant passive leakage of ions by diffusion from healthy plant cells, the possibility cannot be excluded that solutes are being transported outwards from vacuoles by a process which depends either directly or indirectly on respiratory energy, and cannot therefore be detected when metabolism is inhibited. That root cells, in some circumstances, will allow the transport of previously accumulated ions away into the shoot was demonstrated by Steward *et al.* (1942), and the active movement of cations in both directions through the membranes of erythrocytes is well established (Solomon, 1952).

This possibility was examined, with beet cells under the present experimental conditions, by observing the rate of exudation from disks at 25° C. of previously accumulated ^{42}K. Table 2 shows the changes which were observed in the radioactivity of the external medium, when disks, which had been equilibrated with 0·02M-KCl containing ^{42}K, were transferred either to distilled water or to an inactive KCl solution at 25° C. during 6 and 24 hr. Corrections have been made for the decay of the isotope during the experimental period.

Table 2. *Radioactivity (counts per min.) of the external medium, after* 6 *and* 24 *hr. when disks, which had previously been allowed to accumulate* ^{42}K, *were transferred to either distilled water or* 0·02M-KCl, *at* 25° C.

Time (hr.)	Total initial activity of the material	Activity of the medium	
		H_2O	0·02M-KCl
0	2460 ± 115	—	—
6	—	32 ± 4	105 ± 16
24	—	27 ± 3	119 ± 11

The data show that a negligible amount of the previously accumulated ^{42}K left the tissue, when the disks were suspended in distilled water, even during 24 hr., thus confirming a similar observation of Jenny & Overstreet (1939) with excised barley roots. A greater increase in radioactivity was detected when KCl was present in the external medium, indicating that a certain amount of exchange occurred, but the extent of this was clearly limited since the amount exchanged after 24 hr. was not significantly greater than after 6 hr. In order that isotopic equilibrium between the tissue and the medium should be established, it may be calculated that about 43 % of the ^{42}K originally present in the disks should have passed into the external solution. The fact that only about 5 % was exchanged indicates that, if there was any metabolic transport of ions from the material, it was proceeding extremely slowly. It is probable that all the ^{42}K which appeared in the medium was derived from the cell protoplasts, and that vacuolar ions were not significantly involved.

This conclusion was confirmed by a study of the exchange of ^{42}K when disks, which had been allowed to equilibrate with inactive KCl solution, were transferred to a medium containing the isotope. The experiment showed that between 400 and 500 μg. of potassium, out of a total of more than 5000 μg./g. fresh weight of the material, was readily exchanged within 6 hr. at either a high or low temperature. The amount exchanged in this experiment was thus about the same as that which exchanged in the washed tissue capable of absorbing ions rapidly, although the total potassium content of the disks in the present case was more than twice as great (Table 1). It is evident therefore that cell protoplasts may contain only a limited amount of easily exchanged potassium, and this is independent of the total cation content of the material.

So far attention has been confined to the absorption of potassium, but the study has also been extended to include sodium. These two ions together comprise about 90% of the total inorganic cations in the material which we have examined, and of the two there is a considerable excess of potassium. In one case, which may be regarded as typical, there were present in the disks, immediately after cutting, about 2·25 mg. of potassium, and 0·55 mg. of sodium/g. fresh weight of tissue. When washed beet tissue was placed in a mixture of KCl and NaCl containing equal quantities of each, the two cations were absorbed at approximately equal rates, and appeared to compete with one another on more or less equal terms, for the use of the same accumulation mechanism. Absorption eventually ceased when the total concentration of both ions together was about the same as that which was attained when absorption occurred from a solution of either KCl or NaCl alone (Table 3).

When disks, which had been allowed to equilibrate with 0·02 M-NaCl, were transferred to a solution of 0·02 M-KCl, a limited uptake of potassium

Table 3. *Potassium and sodium contents (mg./g. fresh weight) of disks allowed to equilibrate with* (*a*) 0·02 M-KCl, (*b*) 0·02 M-NaCl, (*c*) 0·01 M-KCl + 0·01 M-NaCl, *at* 25° C. *for* 7 *days*

Medium	Cation content		
	K	Na	K–Na
0·02 M-KCl	5·75 ± 0·38	0·53 ± 0·02	6·28
0·02 M-NaCl	1·53 ± 0·08	5·13 ± 0·32	6·60
0·01 M-KCl + 0·01 M-NaCl	4·15 − 0·27	2·38 ± 0·18	6·53

was observed within 24 hr., which was accompanied by a corresponding output of sodium. This may be interpreted as representing the replacement of some sodium in cell protoplasts by potassium. Subsequently there was no significant change in either the potassium, or the sodium content of the disks, although the experiment was prolonged for a further 7 days (Fig. 3). When the disks were treated first with KCl and then placed in NaCl, the

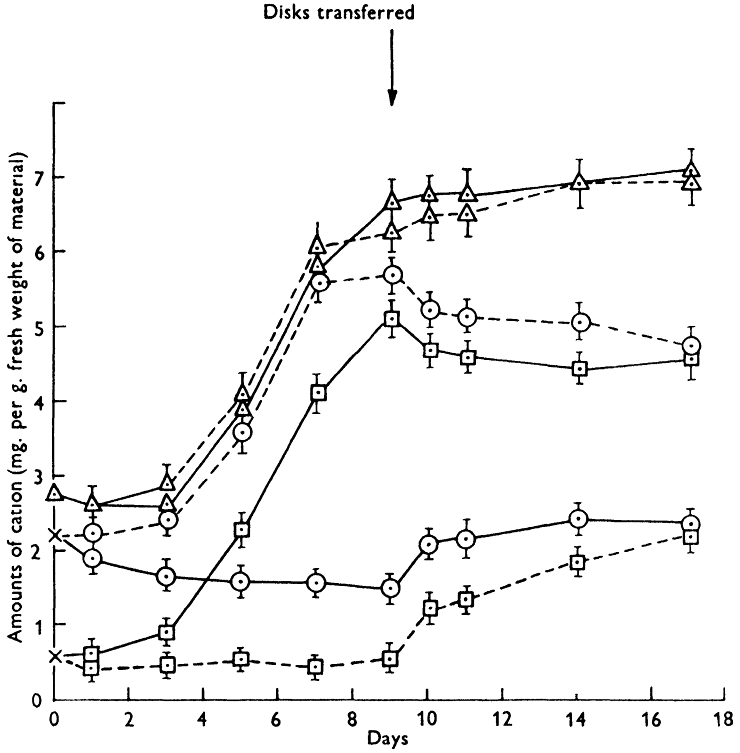

Fig. 3. Amounts of K (⊙), Na (⊡) and Na+K (△) in beet disks placed in 0·02 M-KCl (– – – –) or 0·02 M-NaCl (———) for 9 days at 25° C., and then transferred to the alternative medium for a further 8 days at 25° C.

effects observed were substantially the same. In this case, however, following the preliminary exchange, the sodium content of the tissue continued to increase throughout the experimental period. This was probably due to the fact that the material was not completely equilibrated at the time of transfer, since the total cation content of the disks also increased. The experiment as a whole confirms the view that most of the cations in beet cells, whether potassium or sodium is considered, do not exchange readily over relatively long periods of time, and that the movement of ions between the external medium and cell vacuoles is probably very slow.

It is also evident from the above experiment, that in the presence of a high internal concentration of either potassium or sodium, the absorption of the alternative ion is retarded. This is contrary to the observations of Humphries (1950, 1951, 1952) that the absorption of potassium by excised root systems is related to the amount of this ion which is present in the tissue, and is independent of any other. Humphries grew potassium-deficient plants by replacing this ion in the culture medium by sodium, and found that the excised roots of these plants subsequently absorbed more potassium than the controls. This discrepancy may be resolved, if excised roots, unlike beet disks, selectively absorb potassium rather than sodium. It would be interesting to know the sodium content of these potassium-deficient plants to establish this point.

Since neither a passive leakage nor a metabolic leakage of ions from cells can adequately account for the equilibrium which is established with beet tissue, an alternative hypothesis may be proposed. Hoagland & Broyer (1936) suggested that the accumulation of ions in high-salt barley roots might eventually cease, because of the progressive saturation of protoplasmic constituents. This theory has been developed by Overstreet, Jacobson & Handley (1952) as a part of their theory of the mechanism of ion absorption in roots. They have proposed that uptake depends on the presence in the protoplasm of metabolically produced carrier substances, designated HR, which combine with the ions, e.g. M^+, and then subsequently break down again according to the reversible reaction

$$HR + M^+ \rightleftharpoons MR + H^+.$$

In this reaction, one of the rate-determining factors is the concentration of the complex MR. It is suggested that the level of MR may be higher in high-salt than in low-salt tissues, so that a smaller amount of HR is available to combine with more M^+ from the external medium, and the rate of absorption is reduced.

In extending this hypothesis somewhat, it may be proposed that the effect of a high internal salt concentration is to prevent the breakdown of the ion-carrier complex at the inner surface of the tonoplast of the plant cell, and this may perhaps be associated with the increased activity gradient across which the ions are being moved. It is possible that when ions have been adsorbed on the carrier, this complex is metabolized to a high-energy form, which can then be decomposed to release the ions, as long as the free energy of the whole system is thereby reduced. When equilibrium is attained in the cell, owing to the increased free energy of potassium ions in the vacuole, this condition may be no longer satisfied, so that the ion and its carrier remain in the combined form. No carrier is therefore available to accept ions from the medium, and accumulation stops.

In this study, therefore, the failure both of freshly cut disks and of salt-saturated tissue to accumulate ions has been considered, and in each case it is possible that this may be related to the lack of available carrier molecules. With freshly cut tissue, the carrier is either absent from the protoplasts, or else it is inactivated by an unknown mechanism, whilst with cells which have already absorbed ions to their maximum capacity, the carrier is probably present entirely in the combined form. In either of the two situations, absorption is prevented.

V. THE INTERRELATIONSHIP BETWEEN ABSORPTION OF ANIONS AND CATIONS

So far it has been assumed that a metabolic mechanism is directly involved in the absorption of cations, but in the accumulation of neutral salts the uptake of one ion cannot be divorced from that of its associate. The active absorption of cations automatically creates an electrical gradient along which a passive movement of anions may occur, and vice versa. With many types of animal cell it appears that cations are metabolically transported, whilst the anions follow passively. On the other hand, Lundegårdh (1940) has claimed for plants that anions are actively absorbed, and cations move in along the electrical gradient so created.

The evidence presented above shows that the protoplast of a plant cell presents a very considerable barrier to the free diffusion or exchange of cations, indicating that the presence of an electrical gradient alone may not be sufficient to account for the absorption of K and Na at the rates which have been observed. It seems probable therefore that a specific accumulation mechanism for cations exists in these cells. The inquiry may then be made whether in fact the anions are absorbed passively and the scheme of Lundegårdh be reversed. Experiments with ^{82}Br along the lines of those outlined above for ^{42}K have shown, however, that there is a similar lack of exchangeability between vacuolar anions and the medium, so that for the present it must be concluded in agreement with Hoagland & Steward (1939) that both anions and cations are accumulated directly by a metabolically controlled mechanism. Whether it is the same or a different carrier system which is involved in the two cases must remain the subject of further investigation.

VI. SUMMARY

The complexity of the experimental situation with growing cells and tissues is indicated, and the importance of attempting to study ion-absorption in non-growing cells is emphasized. It is proposed that the development of a capacity to absorb solutes in beet disks may be related to the metabolic synthesis, or activation of carrier substances in the tissue. Evidence is presented that combination between the ion and its carrier occurs in the protoplasm by a non-metabolic mechanism, and that active transport takes place across the tonoplasts. It is probable that the physical resistance of these membranes to diffusive processes is very high, and that exchanges of ions between cell vacuoles and the external medium are prevented. The establishment of an equilibrium, when accumulation ceases in non-growing cells, may be attributed to a failure of the ion-carrier complex to break down when the activity gradient across which the ions are moving becomes too great.

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THE RELATIONSHIP BETWEEN METABOLISM AND THE ACCUMULATION OF IONS BY PLANTS

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I. INTRODUCTION

A discussion of the probable nature of the mechanism whereby ions are accumulated in plant tissues must turn largely on two fundamental questions which are still matters of controversy, namely:

(1) Is the cytochrome-cytochrome oxidase system the only mechanism able to make energy available for the active accumulation of electrolytes in plant tissues or do other terminal oxidases share this characteristic?

(2) Is the accumulation of ions directly mediated by the electron transfer in respiration or does a product of respiration function as an ion-carrier?

The investigation of the former subject to be described in this paper was undertaken jointly with Dr W. O. James of the Department of Botany, Oxford University. The remaining experiments were carried out in the Department of Agriculture, Oxford University, in collaboration with Dr R. P. Martin, Miss Joyce Ayland and others.

It is a pleasure to acknowledge my indebtedness to those who have collaborated in this work and also to Dr J. L. Harley and Dr J. F. Sutcliffe who have both made unpublished data available to me.

II. THE ABILITY OF TERMINAL OXIDASE SYSTEMS OTHER THAN CYTOCHROME TO MEDIATE ACTIVE ACCUMULATION

Lundegårdh (1945, 1950) and Robertson & Wilkins (1948) have concluded that the active accumulation of ions is dependent upon respiration mediated by cytochrome oxidase. Their results suggest that in wheat roots and carrot slices other terminal oxidases may be without effect in this respect. However, as cytochrome appears to be a principal path of respiration in the tissues they investigated, it would seem rash to conclude that the same result would be obtained in tissues possessing different terminal oxidase systems. Thus it was decided to examine the relationship between respiration and salt uptake in material in which a large fraction of the normal respiration is mediated by systems other than cytochrome. The roots of young barley plants were suitable for this investigation, since, at certain stages of their development, ascorbic acid oxidase is the principal terminal oxidase (James, 1953). Grain of the variety Spratt Archer was germinated on moist cotton gauze supported above distilled water at room temperature. Apical 10 mm. segments of root were detached for experimental treatment after varying periods of growth. Respiration was determined by the standard Warburg procedure, and the absorption of bromide and rubidium was measured by tracer methods, both the root apices and the external solution being assayed. The duration of each experiment was 3 hr. Diethyldithiocarbamate, which chelates copper (Albert & Gledhill, 1947), was employed as a respiratory inhibitor. James & Garton (1952) have shown that at the concentration of 2×10^{-4}M this substance inhibits the ascorbic acid oxidase of barley to an extent of over 85 %; by contrast the inhibition of cytochrome oxidase is less than 10 %. It was found (James, unpublished) that the extent to which the respiration of barley root apices is affected by 2×10^{-4}M-diethyldithiocarbamate varies with the age of the tissues (Fig. 1). Three days after germination this inhibitor reduced respiration by approximately 35 %; thereafter the effect increased until in the apices of roots 7 days old respiration was inhibited to over 60 %. Concurrently with the increasing sensitivity of the tissues to diethyldithiocarbamate, the extent of light-reversible inhibition by carbon monoxide fell. The respiration of recently germinated embryos was inhibited to the extent of 80 % by carbon monoxide, whereas when roots had reached the age of 7 days virtually no inhibition occurred. The complexity of the pattern of respiration in this tissue is further indicated by that fact that, in root

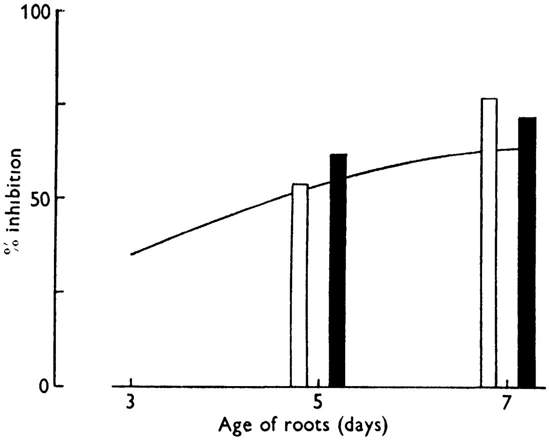

Fig. 1. Effects of 2×10^{-4}M-diethyldithiocarbamate in the apical 10 mm. of barley roots. Curve—respiration. Histograms: unshaded—absorption of rubidium; shaded—absorption of bromide.

apices exceeding 7 days in age, the effect of diethyldithiocarbamate declined.

A general discussion of the balance of terminal oxidase aystems is beyond the scope of the present inquiry, and consideration will be here confined to the relationship between respiration and salt uptake in roots 5 and 7 days old. The effect of 2×10^{-4}M-diethyldithiocarbamate on the absorption of bromide and rubidium from 0·002M-solutions is shown by the histograms superimposed on Fig. 1. It is apparent that the effects of this inhibitor on respiration and on salt absorption were similar. The inhibition of the absorption of both ions rose from between 50 and 60% at the fifth day to over 70% two days later. Since the extent to which 2×10^{-4}M-diethyldithiocarbamate inhibits salt uptake greatly exceeds the extent to which it inhibits cytochrome oxidase, the observed effect on salt uptake cannot be attributed to the inhibition of the cytochrome system. Further evidence in this direction is provided by results of James's experiments with carbon monoxide which indicate that cytochrome oxidase is responsible for a negligible part, if any, of the respiration of roots 7 days old. A clear relationship between the inhibition of ascorbic acid oxidase and the inhibition of salt uptake is instead indicated.

Table 1. *The effect of diethyldithiocarbamate on the absorption of rubidium and bromide by barley plants at the second leaf stage*

Ion	Concentration of diethyldithio-carbamate (molar)	Percentage reduction of absorption induced by inhibitor	5 % fiducial limits
Rb, 10^{-3}M	2×10^{-4}	69·9	±2·9
Br, 8×10^{-3}M	2×10^{-4}	67·2	±0·8
	10^{-3}	73·7	±1·8

Additional evidence that respiration through the ascorbic acid oxidase system can provide energy which is utilized in salt absorption was obtained in experiments in which intact barley plants at the second leaf stage were treated with 2×10^{-4}M-diethyldithiocarbamate (Table 1). The absorption of both rubidium and bromide was inhibited to between 65 and 70%. When the concentration of diethyldithiocarbamate was increased to 10^{-3}M, salt absorption was decreased to a small but significant extent. James & Garton (1952) have shown that this concentration completely inhibits ascorbic acid oxidase and reduces the activity of cytochrome oxidase by upwards of 25%. It is possible therefore that a small fraction of the salt absorption of the plants was mediated by cytochrome. Some further evidence of the relationship between ascorbic acid oxidase and salt absorption is provided by an experiment to which reference is made later (Fig. 4), in which 10^{-4} M-diethyldithiocarbamate inhibited the absorption of phosphate by barley plants to the extent of over 30 % when the external concentration of phosphate was 10 p.p.m.

Having shown that respiration mediated by ascorbic acid oxidase can bring about the active accumulation of salts, it is natural to inquire if this capacity is common to all terminal oxidases, or whether metallo-enzymes alone possess it. In this connexion interest attaches to the recent investigations of Harley and his associates (1953) on the nutrient absorption and metabolism of beech mycorrhizal roots. In fresh excised mycorrhizas (Table 2) it was found that cyanide, azide, fluoride iodoacetate and arsenate had no inhibiting effect on oxygen uptake, though they inhibited phosphate uptake to a marked extent. Malonate at the concentration used had no significant effect on respiration or absorption. The respiration rate was unchanged by the addition of salts to the external medium. Tissues which had been stored in distilled water gave contrasting results; a marked 'salt respiration' was induced by 64×10^{-3} M-KH_2PO_4 (Table 3). Cyanide, surprisingly, also stimulated respiration, and when cyanide and phosphate were applied together a still greater increase was observed. Phosphate absorption was reduced by cyanide to the extent of approximately 50 %, a comparable degree of inhibition to that in fresh roots.

Table 2. *Effect of inhibitors on the absorption of oxygen and phosphate by fresh, excised beech mycorrhizas at* pH 5·5

Harley, McCready & Brierley (1953).

	% of control	
	O_2	P
Cyanide 10^{-3} M	106	49
Azide 2×10^{-5} M	96	50
Iodoacetate 10^{-3} M	100	48
Fluoride 4×10^{-2} M	97	35
Malonate 5×10^{-2} M	90	100
Arsenate 10^{-3} M	99	39

Table 3. *Effect of* 5×10^{-3} M-KCN *and* 64×10^{-3} M-KH_2PO_4 *on the oxygen uptake of beech mycorrhizas after storage in distilled water*

(Data from Harley, McCready & Brierley, 1953.)

Treatment	% of control	
	Exp. (i)	Exp. (ii)
KCN	164	190
KH_2PO_4	170	179
KCN + KH_2PO_4	192	241

No detailed interpretation of these puzzling results appears at present possible; it can only be concluded that there is no simple relationship between the activity of metallo-terminal oxidases and the accumulation of phosphate in this tissue. This being so it is probable that respiration through a flavo-protein system was associated with salt uptake.

III. THE LINK BETWEEN RESPIRATION AND SALT ACCUMULATION

In view of the recent reviews by Broyer (1951) and Overstreet & Jacobsen (1952) it is unnecessary to discuss in general terms the theories which have been advanced to explain the active accumulation of salts. It is widely agreed that ionic exchange processes play an important part in the initial entry of ions into the cytoplasm. But the conflict of opinion is sharp with regard to the subsequent steps in the transfer of ions across the cytoplasm to the vacuole or the vascular stele. The 'anion respiration' concept developed by Lundegårdh and by Robertson and his associates is well known. The distinctive features of this postulate are that the movement of anions across the cytoplasm is directly mediated by the electron transfer through the cytochrome-cytochrome oxidase system, while cations move by diffusion. The alternative mechanism most favoured in recent years postulates a product of respiration or 'carrier' whereby ions are placed under restraint and are moved against an ionic gradient (Wohl & James, 1942; Jacobsen, Overstreet, King & Handley, 1950).

While the results so far presented are incompatible with Lundegårdh's concept in its present form, they would not conflict with a theory postulating that the electron transfer mediated by all terminal oxidases can promote the accumulation of salts in the manner attributed by him to cytochrome. Such an interpretation would, however, be unsupported by positive evidence, and, furthermore, would be difficult to reconcile with the more recent extension of Lundegårdh's theory (1952) in which it has been necessary to visualize the three cytochromes, *a*, *b* and *c*, as participating in different steps of the mechanism.

Clearly an entirely different experimental approach is necessary if one or other of the postulated mechanisms is to be established and the other rejected. It seemed that some progress in this direction would be made if it could be shown that roots can store a capacity to transfer ions across the cytoplasm by virtue of *prior* respiration; the concept of 'anion respiration' denies this possibility, but if a carrier mechanism is operative it would be expected that at any instant healthy tissues will possess some capacity to accumulate ions by virtue of the carrier within them formed by prior respiration which has not yet mediated the accumulation of ions.

Since such an effect has not been observed hitherto, it is reasonable to infer that, if it does occur, the quantity of ions which can be accumulated in this way is insignificant by comparison with that absorbed under normal experimental conditions. An investigation of this question can therefore be expected to be profitable only if very low concentrations of salt are employed in the external solution; tracer methods make this relatively easy, and the principal difficulty to be overcome is to determine whether the ions entering the plant have in fact been subject to metabolic accumulation. Exchange or adsorption effects on the cytoplasm would be expected to account for a considerable entry under such conditions, and the importance of determining whether or not the absorbed ions have been held in this manner is obvious.

This requirement can be largely satisfied by using intact plants. A number of investigators have concluded that the movement of ions across the symplast to the stele is a process analogous to the movement of ions across the cytoplasm of a single cell to its vacuole (Wiersum, 1947; Lundegårdh, 1950; Arisz, 1951). Thus if experimental periods are so short that the nutrient status of the plant is virtually unaffected by the ions absorbed, changes in the content of shoots may be regarded as reflecting similar changes in the extent to which ions are transferred across the cytoplasm. The principal drawback of such studies is the labour they entail, and the considerable magnitude of experimental errors. Simpler material, such as detached roots or tissue slices, cannot, however, provide equally unequivocal evidence of active transport.

An investigation of the absorption of phosphate by young barley plants provided the opportunity for examining this question. Plants were grown to the second leaf stage in a solution containing a balanced supply of nutrients other than phosphate. The phosphate present in the seed was sufficient to prevent visual symptoms of deficiency, and the resultant plants showed a considerable capacity for phosphate accumulation. Before discussing the evidence which these investigations provide on the mechanism of nutrient accumulation, it is necessary to summarize data, some of which has been presented elsewhere (Russell & Martin, 1953; Russell, Martin & Bishop, 1953*a*) regarding the effect of the amount of phosphate entering plants on its distribution between roots and shoots, and the relationship between the external concentration and the rate of absorption of phosphate.

When plants were treated for 24 hr. with concentrations of labelled phosphate ranging from 10^{-8} to 10^{-4}M-H_2PO_4 (i.e. 0·000316 to 3·16 p.p.m. P), the fraction of the absorbed phosphate which was found in the shoots increased progressively as the external concentration of phosphate was increased (Fig. 2, curve *A*). Still higher concentrations caused the

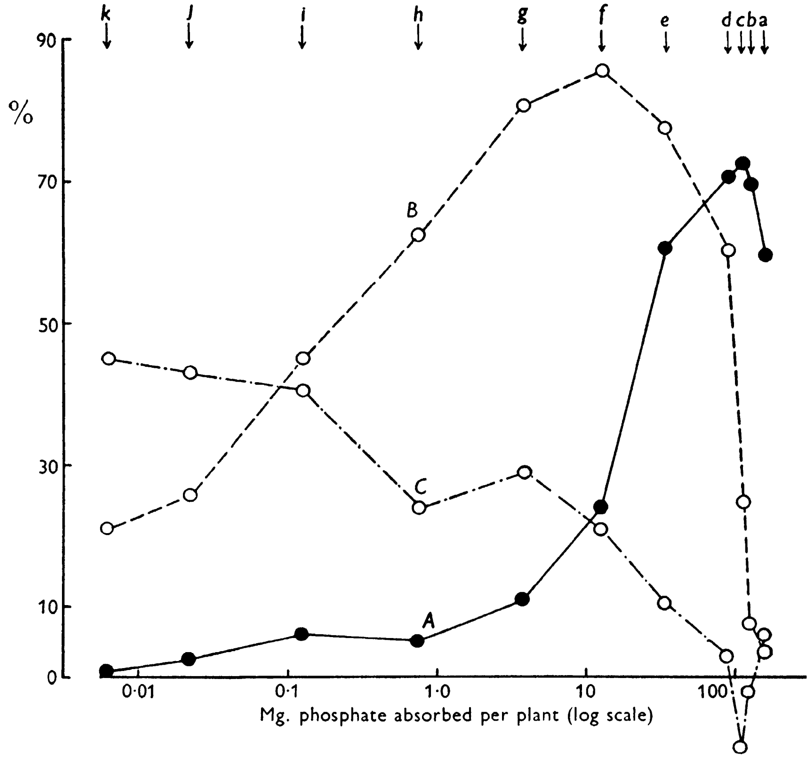

Fig. 2. The absorption, distribution and subsequent loss of phosphate by young barley plants treated with different concentrations of labelled phosphate for 24 hr. Concentration of labelled phosphate applied (p.p.m. P). *a*, 31·6; *b*, 10·0; *c*, 3·16; *d*, 1·00; *e*, 0·316; *f*, 0·100; *g*, 0·0316; *h*, 0·0100; *i*, 0·00316; *j*, 0·001; *k*, 0·000316. Curve *A*, percentage of absorbed phosphate in shoots. Curve *B*, relative absorption. Curve *C*, percentage of absorbed phosphate lost by plants transferred to phosphate free solutions for 7 days.

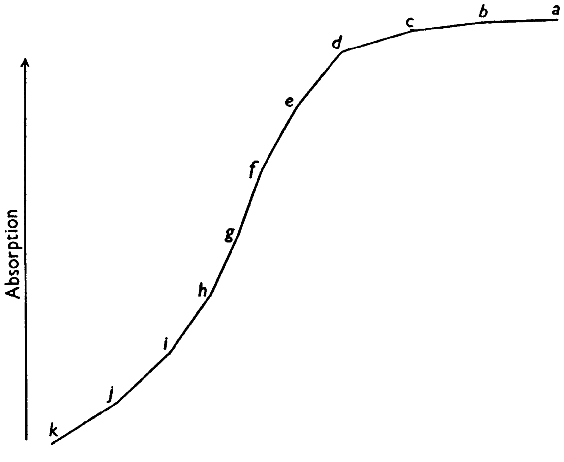

Fig. 3. Relationship between absorption and exponential mean external concentration for the same experiments as Fig. 2. (Co-ordinates not to scale, see text.)

proportion of phosphate in the shoots to decrease; between experiments the concentration which induced this effect varied. The initial phosphate content of the plants was in the order of 0·15 mg., and it is apparent from Fig. 2 that over the greater part of the concentration range, the phosphate content of the plants was changed to a negligible degree during the course of the experiments. Thus differences in the distribution of the absorbed phosphate cannot be attributed to effects on the rate of growth of the plants caused by the nutrient absorbed during the experimental periods. Moreover, the interaction of phosphate with other ions simultaneously absorbed cannot explain the changing pattern of its distribution, since the same general effect was observed when phosphate was supplied alone, or in the presence of other nutrients.

An examination of the relationship between the external concentration of phosphate and the rate of absorption is complicated by a number of circumstances resultant on the low concentrations of phosphate employed. Evidence to be presented elsewhere (Russell, Martin & Bishop, 1954) shows, however, that the general nature of the effect of external concentration on the rate of absorption can be validly assessed. The wide range of concentrations employed make it impossible to represent the relationship between absorption and the exponential mean concentration in a graph of manageable size, using a linear scale. As in the present discussion interest centres on changes in the slope of the curve, it is sufficient to consider a diagrammatic representation (Fig. 3) in which the vertical and horizontal scales have been varied concurrently, so that the lines joining adjacent points are of equal length, but their slope is correctly shown. A sigmoid relationship between absorption and the external concentration is apparent. The region of maximum slope occurred when the external concentration was between 0·1 and 0·03 p.p.m. P. The steady decrease in the angle of the curve below this region indicates that the amount of phosphate absorbed *decreased more rapidly* than the external supply of phosphate when the external concentration was lowered. The same relationship is shown by calculated values of 'relative absorption' in which the phosphate absorbed is expressed as a percentage of the amount available to the plants; the values show a well-marked maximum for plants supplied with 0·1 p.p.m. P (Fig. 2, curve *B*).

This relationship is strikingly at variance with the proportionality between external concentration and absorption which normally obtains in dilute solutions. A possible explanation is suggested by other observations. When plants which had been treated with varying concentrations of labelled phosphate for 24 hr. were transferred to phosphate-free media, the proportion of the phosphate which had been absorbed in the previous period which was lost from the plants to the external solution was greatest in the plants which had been supplied with the most dilute solutions (Fig. 2, curve *C*). It is apparent from Fig. 2 that plants supplied with less than 0·1 p.p.m. P showed a marked increase in phosphate loss, while the proportion of the absorbed phosphate found in the shoots and relative absorption both declined. This suggests that smaller fractions of the absorbed phosphate which were found in the shoots of plants supplied with low concentrations of phosphate, and their reduced efficiency of absorption, were due to phosphate being retained by some mechanism in the roots which subsequently released it to the outer medium. Phosphate retained in this manner had clearly not equilibrated with the phosphate already present in the plants; this was shown by the fact that no significant loss of phosphate occurred from plants which were grown to this stage in the absence of external sources of that nutrient.

In seeking an interpretation of these effects, consideration was first given to whether the observed retention of phosphate in roots was due to a metabolic process or to a physical mechanism such as adsorption on inert surfaces. If retention were due to adsorption on inert surfaces, the extent of depletion of the solution, and therefore the rate of relative absorption, would be expected to be greatest when the lowest concentrations of phosphate were provided. This was not so. The loss of labelled phosphate when plants were transferred to phosphate-free media is also difficult to reconcile with the view that the phosphate had been retained by a simple physical process. Further evidence regarding the nature of the mechanism of phosphate retention was obtained by pretreating plants for 24 hr. with unlabelled phosphate of concentration 10 p.p.m. In this manner their phosphate content was increased by approximately 65%. Pretreatment had little effect on the absorption of labelled phosphate from solutions containing 0·001 or 10 p.p.m. P during the subsequent 24 hr., nor was the distribution of phosphate between roots and shoots significantly affected in the plants supplied with 10 p.p.m. P. When, however, the external concentration of phosphate was 0·001 p.p.m. P, the content of shoots was increased over thirty times by the pretreatment (Table 4). An effect of the same type, though of lesser magnitude, was shown when plants of considerably greater age were similarly treated.

The foregoing results are most readily interpreted as indicating that metabolic processes in the root retain phosphate against upward movement, and that the proportion of the entering phosphate which is retained in this way is greatest when the phosphate content of the roots is low. Thus it appears that the distribution of phosphate between the roots and shoots of barley plants reflects the interaction of two metabolic processes, namely, that responsible for retention in the roots and that responsible for the transference of phosphate to the stele.

Table 4. *Effect of pretreatment with phosphate* (10 *p.p.m.* P) *for* 24 *hr. on the absorption and distribution of labelled phosphate in young barley plants during the subsequent* 24 *hr.*

When a logarithmic transformation was necessary for statistical analysis, the transformed values are shown in italics.

Initial concentration of solution (p.p.m. P)		μg. P per plant			% of absorbed phosphate in shoots
Pre-treatment	Treatment	Root	Shoot	Total	
Nil	10	39·1	51·9	91·8	55·8
10	10	42·0	52·5	94·5	55·5
S.D.		n.s.	n.s.	n.s.	n.s.
Nil	0·001	0·0647	0·0004 *(0·604)*	0·0651	0·63 *(0·799)*
10	0·001	0·0649	0·0149 *(2·173)*	0·0798	18·80 *(2·274)*
S.D.		n.s.	*(0·163)*	n.s.	*(0·152)*
Phosphate absorbed during pretreatment		43·4	55·0	98·4	56·0

Experiments with respiratory inhibitors enable some conclusions to be drawn with regard to these two mechanisms. Typical results for experiments lasting 24 hr. are summarized in Fig. 4. At the highest concentration of phosphate (i.e. 10 p.p.m. P), the application of a range of concentrations of sodium azide, 2, 4-dinitrophenol and diethyldithiocarbamate markedly reduced absorption, the content of the shoots being depressed to a greater extent than that of the roots. When, however, the initial concentration of the external solution was 0·001 p.p.m. P, the phosphate content of the shoots of plants at the second leaf stage was increased by certain concentrations of the inhibitors; 10^{-3}M-sodium azide, 10^{-4}M-diethyldithiocarbamate and 10^{-5}M-2, 4-dinitrophenol were most effective in this regard. The phosphate content of the roots of plants which showed this effect was in some experiments increased though to a lesser extent than that of the shoots; in other experiments it was reduced but to a smaller extent than in plants supplied with 10 p.p.m. P. Higher concentrations of the inhibitors reduced the content of both roots and shoots.

As in all studies conducted in a greenhouse in which the vagaries of climate are not fully controlled, there was considerable variation between different experiments. It was shown in experiments with 10^{-3}M-sodium azide, the inhibitor most extensively used, that the nature of the effect of the inhibitor on the phosphate content of the shoots of plants supplied with 0·001 p.p.m. P varied with plant age (Fig. 5). When plants of which the first leaf was developing were used, the inhibitor reduced the phosphate content of shoots, though the magnitude of the

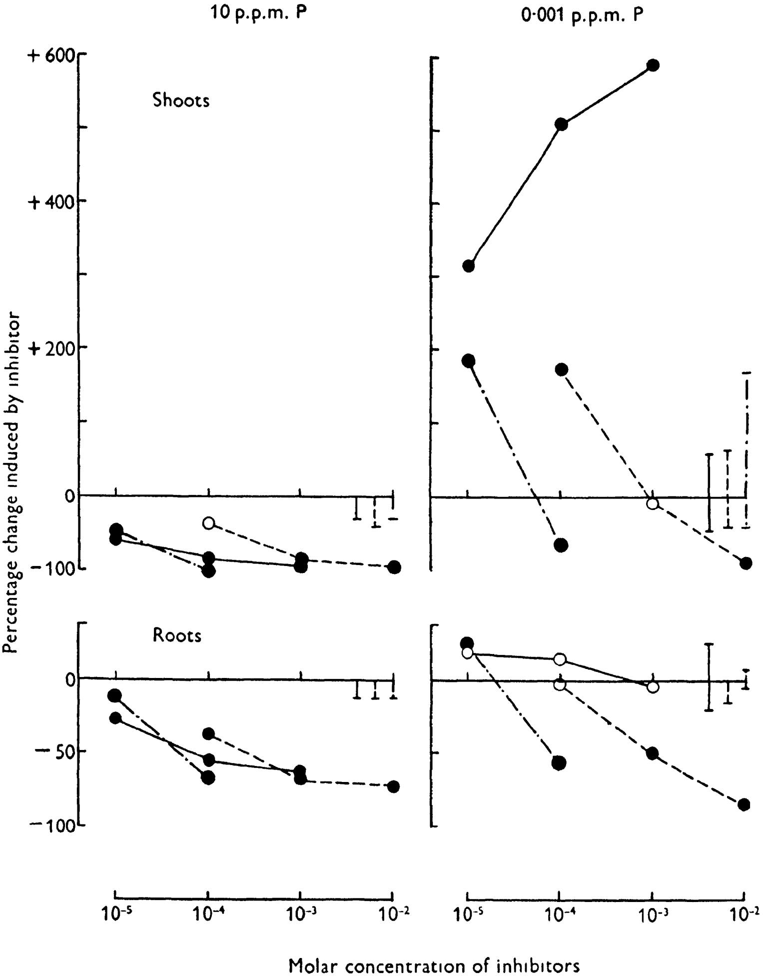

Fig. 4. Changes in phosphate content of roots and shoots of young barley plants induced by inhibitors in experiments lasting 24 hr. Left: Concentration of phosphate in external solution 10 p.p.m. P. Right: Concentration of phosphate in external solution 0·001 p.p.m. P. Solid lines: sodium azide. Broken lines: diethyldithiocarbamate. Dot-dash lines: 2,4-dinitrophenol. Values expressed as percentages of controls. Significant differences from control treatments are shown and significant effects are indicated by solid circles.

effect was less than in plants supplied with 10 p.p.m. P. When progressively older plants were similarly treated, increasing stimulation of phosphate transfer to the shoots were shown. This result indicates that the effect of the inhibitor in increasing the content of shoots was not a necessary consequence of a small amount of phosphate entering the plant. A comparison of the curves for the percentage change in shoot content induced by

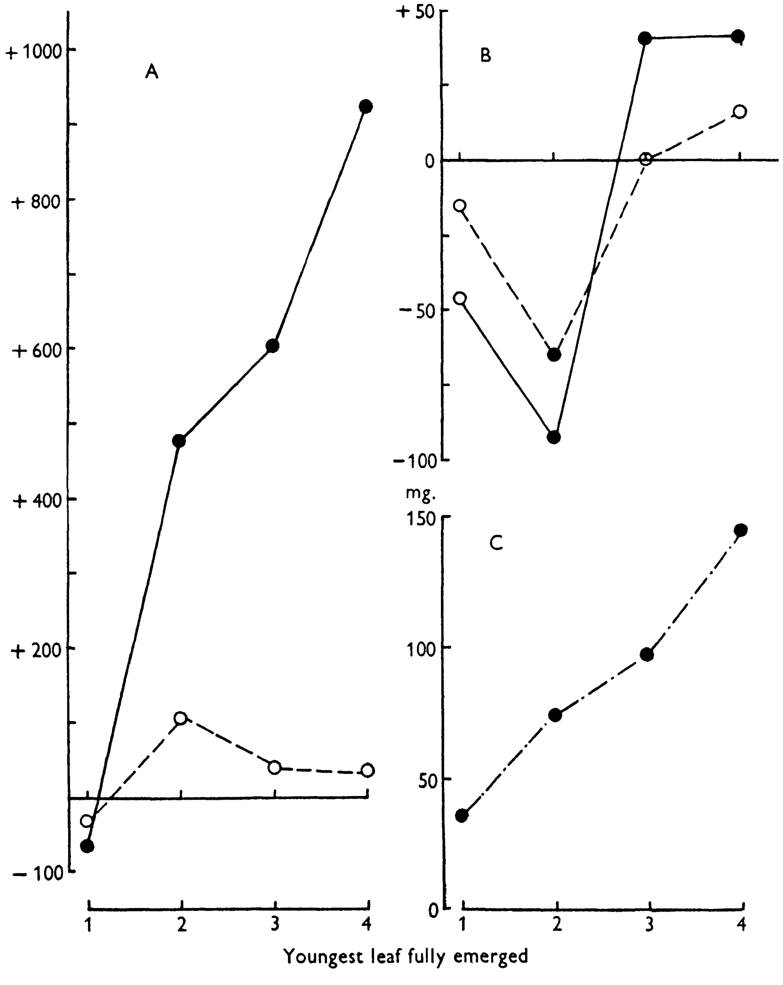

Fig. 5. Effect of sodium azide on the absorption of phosphate by barley plants of different ages from solutions containing 0·001 p.p.m. P, in experiments lasting 24 hr. A and B, changes in phosphate content of shoots and roots respectively. Solid line: 10^{-3}M sodium azide. Broken line: 10^{-5}M sodium azide. Significant effects are indicated by solid circles. C, mean dry weight of control plants.

10^{-3}M-azide (Fig. 5A) and for plant dry weight (Fig. 5C) shows an obvious similarity. The relationship between these quantities was examined on the pooled data of this and two other experiments, and a highly significant linear regression of plant dry weight on the percentage increase in shoot content induced by 10^{-3}M-azide was demonstrated, the equation being

$$y = (0{\cdot}102 \pm 0{\cdot}021)\, x - 21{\cdot}4.$$

Since the plants received no phosphate before the experimental treatment, their phosphate status varied inversely with their weight. On the basis of the results obtained by pretreating plants with phosphate (Table 2), it is to be expected that plants of low phosphate status will show the greatest metabolic retention of phosphate in roots. Thus the observed relationship between plant weight and the effect of the inhibitor is regarded as indicating that the extent to which shoot content is increased by the inhibitor depends on the extent to which phosphate entering the plant would be metabolically retained in the roots if no inhibitor were present. It is therefore considered that the primary effect of the inhibitor was in all probability the inhibition of phosphate retention, and that the stimulation of phosphate transfer to the shoots was an indirect effect due to a larger fraction of the entering phosphate being available for transference to the stele.

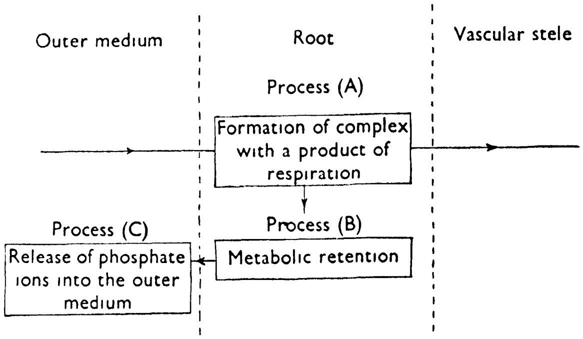

Fig. 6

The results of these experiments can be explained in terms of the three interrelated processes shown in Fig. 6. Each process, in all probability embraces a number of stages: *Process A*: the formation at or near the cell surface of a complex between the entering ion and a product of respiration; this process is envisaged as resulting in the ultimate release of the ion into the stele, unless it is diverted into *Process B*, the mechanism of metabolic retention in the cytoplasm of the root. *Process C* results in the ultimate release into the outer medium of a fraction of ions which have previously been utilized in process B. It is postulated that the root possesses a certain capacity to accomplish process A in consequence of the necessary metabolic product having been produced by prior respiration. Thus the inhibition of respiration will bring about a comparable inhibition of process A only if the amount of respiratory intermediate is negligible by comparison with the number of ions available for absorption. The extent to which phosphate is utilized in process B is, however, regarded as dependent on the requirements of concurrent metabolism. It is postulated further that process B has a greater affinity for phosphate than process A. Thus, if the amount of phosphate entering the plant is reduced so that it is insufficient to saturate both mechanisms, the proportion of the phosphate diverted to process B will be increased. This will in turn increase the extent of process C relative to process A. In this way the reduction in the efficiency of absorption which occurred when the external concentration was reduced below 0·1 p.p.m. P can be explained.

The results of the experiments with inhibitors are also compatible with the proposed mechanism. When the external concentration of phosphate was high (10 p.p.m. P) absorption and translocation to the shoot was greatly reduced by inhibitors, as would be expected if the rate of process A was closely related to the rate of simultaneous respiration. When the external concentration of phosphate was low (0·001 p.p.m. P) and the extent of metabolic retention in the plants not treated with inhibitors was also low, absorption and translocation were reduced by inhibitors to a much smaller extent; this is attributed to the fact that the product of prior respiration was now able to mediate the absorption of a significant proportion of the ions available to the plants. When, however, the external concentration of phosphate was low (0·001 p.p.m. P) and the extent of metabolic retention in the plants not treated with inhibitors was high, upward movement to the shoot was increased by inhibitors, and in some cases the total amount of phosphate absorbed was increased also. The increased shoot content is interpreted as indicating that the extent of inhibition of process B sufficiently exceeded that of process A to cause a greater rate of release of ions into the vascular stele. The increase in total plant content induced by inhibitors in some experiments suggests that phosphate loss through process C was reduced to a greater extent than primary absorption (process A).

The speculative nature of this interpretation is obvious. The results, however, appear irreconcilable with any theory which denies the ability of roots to 'store' the capacity to transfer ions across the cytoplasm. The concentrations of sodium azide and diethyldithiocarbamate which increased the movement of phosphate to the shoots of plants supplied with 0·001 p.p.m. P have been shown to inhibit respiration; thus the concept that the electron transfer in respiration directly mediates the accumulation of ions is unacceptable. The formation of a product of respiration which serves as a carrier must therefore be envisaged. It is of interest that

Sutcliffe reached a similar conclusion in his study of the absorption of cations by slices of beet, which have been described earlier in this Symposium.

Because of the special role of phosphate in metabolism, results obtained with that ion must be treated with caution in making generalizations regarding absorption. However, the view that information on the relationship between absorption and respiration gained by the use of phosphate may be of general application is encouraged by the fact that respiratory inhibitors appear to affect the absorption of different ions from relatively concentrated solutions to apparently the same extent. In a series of experiments in which 10^{-3}M-diethyldithiocarbamate was applied to barley root tips, the percentage inhibition of the absorption of phosphate and of bromide from 0·002 M solutions, together with the 5 % fiducial limits were:

P: 67·3 ± 10·4% inhibition,

Br: 73·3 ± 9·7% inhibition.

IV. THE INTERACTION BETWEEN EXCHANGE PROCESSES AND THE CARRIER MECHANISM

Before considering the inference which can be drawn concerning the probable nature of the mechanism whereby ions are accumulated, it is desirable to consider the part played by ionic exchange in this process. The importance of exchange reactions has been recognized since the earlier investigations of Briggs (1930) and Brooks (1939) and the simultaneous occurrence of ionic exchange reactions and of the movement of ions by the postulated carrier mechanism must be envisaged. Knowledge of the relationship between these two processes is of obvious importance if information concerning the functioning of the carrier mechanism is to be derived from observations of salt uptake by plant tissues. If the two processes are relatively independent one of the other, each may exert a separate influence on the rate of influx of ions into tissues; thus changes in salt uptake induced by experimental treatments may be due to effects on either or both mechanisms. If, on the other hand, the two processes are closely related, as would be the case if all exchangeable ions in the cytoplasm were located on the carrier, changes in the rate of association of ions with the carrier would be indicated by changes in the rate of entry of ions into the tissue. The study of the carrier mechanism would thus be greatly simplified. The results of many investigations suggest that the rates of the two processes vary independently (e.g. Brooks, 1939; Broyer, 1950). Recently, however, Jacobsen *et al.* (1950) have proposed a carrier mechanism which implies that the process of ionic entry by isotopic exchange is identical with the association of the ion with the carrier. They have postulated that binding substances (HR and $R'OH$) are produced by metabolism and that entering ions (M^+ and A^-) react with them at or near the surface of the protoplasm in the following manner:

$$HR + M^+ \rightleftharpoons MR + H^+,$$

$$R'OH + A^- \rightleftharpoons R'A + OH^-.$$

The complexes MR and $R'A$ are considered to transfer the ions to the vacuole in which they are released by the chemical alteration of the complexes. On the basis of this concept, Epstein & Hagen (1952) and Epstein (1952) have discussed the mode of binding of ions in the postulated carrier. They observed the effects of varying external concentrations of potassium and sodium on the rate of absorption of rubidium by detached barley roots, and after a kinetic analysis of their results arrived at the conclusion that potassium competes for the same sites as rubidium in the postulated carrier, while sodium does not. Applying the same procedure to anions, Epstein (1953) concluded that chloride, but not nitrate, is bound at the same sites as bromide. These interpretations rest on the assumption that the effect of one ion on the rate of entry into the cell of another ion of the same sign is due solely to competition for sites in the complex which effects the transference of ions across the cell. Stated otherwise, this means that changes in the concentration of one ion will affect the rate of entry of another ion of the same sign into the cytoplasm, and its rate of transference across the cytoplasm, in the same manner. If these assumptions are valid, the methods of Epstein & Hagen would provide an important approach to the study of the carrier mechanism. Since, however, they did not examine the fate of the ions absorbed by the tissues which they studied, their interpretation lacks proof. Information on this question could be obtained by applying combinations of ions similar to those used by Epstein & Hagen to a system in which it was possible to make separate observations of total absorption and of the metabolic transport of ions across the cytoplasm. For the reasons stated earlier, barley seedlings were considered suitable for the purpose.

It was found that when barley plants at the second leaf stage were treated with combinations of rubidium and potassium, both the absorption of rubidium and its distribution between roots and shoots were markedly affected. Absorption was reduced in a manner comparable to that reported by Epstein & Hagen. The extent and nature of the effect on the distribution of rubidium between roots and shoots, varied dependent on the prior treatment of the plants. Their potassium status was a major factor in this regard.

For the experiment illustrated in Fig. 7 and Table 5 plants were raised to the second leaf stage in a potassium-free nutrient solution of the following composition in milli-equivalents per litre: Mg, 3; Ca, 9; Na, 2; SO_4, 3; H_2PO_4, 1; NO_3, 10. Half the plants were then pretreated for 24 hr. with $0{\cdot}5 \times 10^{-3}$M-potassium, the remainder being maintained in a potassium free medium. 10^{-3} or 10^{-2}M-labelled rubidium was then provided for 4 hr. in the presence and absence of 2×10^{-2}M-potassium. The absorption of rubidium and its distribution between roots and shoots was significantly affected by all single-factor effects and first-order interactions, with the exception that absorption was not affected by the interaction between pretreatment and simultaneous treatment with potassium. From the viewpoint of the present discussion, the effect of potassium on the distribution of rubidium is of particular relevance, and it alone will be discussed. Whereas pretreatment with potassium reduced the fraction of rubidium absorbed in the subsequent period which was moved to the shoots, the reverse effect was induced by potassium in plants which had not been pretreated with that ion when rubidium was supplied at the higher level. Somewhat contrasting results were obtained when the same concentrations of potassium and rubidium were applied to plants which had

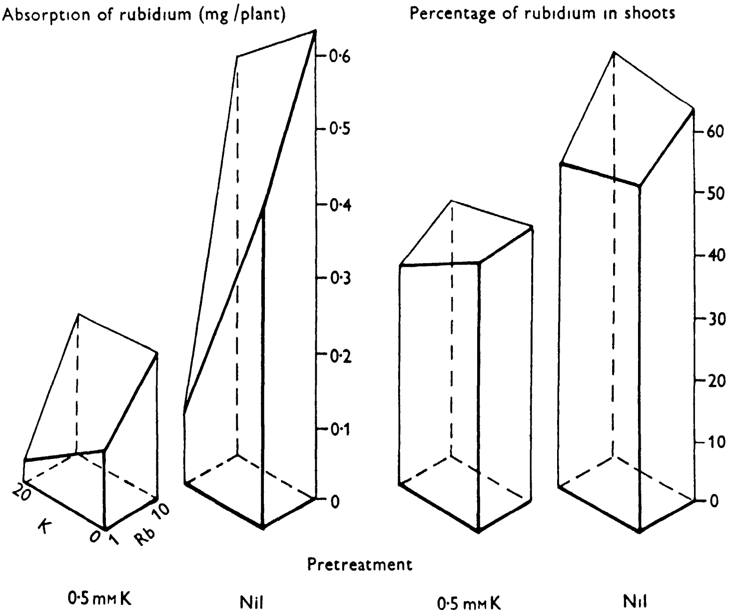

Fig. 7. The absorption and distribution of rubidium in young barley plants to which potassium was provided prior to, or simultaneously with, rubidium. For statistical analysis see Table 5.

been raised in 10^{-3}M-$Ca(NO_3)_2$ and 10^{-3}M-$CaSO_4$. Their rate of growth was greatly retarded as compared with plants grown in the potassium free nutrient solutions. Under these circumstances the presence of potassium significantly increased the proportion of the absorbed rubidium found in the roots at both levels of rubidium (Table 6).

Table 5. *The absorption and distribution of rubidium in young barley plants to which potassium was provided prior to, or simultaneously with, rubidium*

Before the experiment the plants were grown in a potassium-free nutrient solution.

0–48 hr.*	Pretreatment with potassium ($\text{M} \times 10^{-3}$)	0·5		Nil	
72–76 hr.	Concentration of rubidium ($\text{M} \times 10^{-3}$)	1	10	1	10
	Rubidium absorbed mg./plant				
	K absent	1·33	2·33	5·20	7·29
	K present (2×10^{-2}M)	0·32	2·35	1·20	6·15
	% of absorbed rubidium in shoots				
	K absent	43·87	44·04	55·69	63·66
	K present (2×10^{-2}M)	36·90	40·79	52·33	66·82

* From 48 to 72 hr. all plants were in distilled water.

Statistical analysis

Effect	Variance ratios	
	Absorption† of rubidium (mg.)	% of absorbed rubidium in shoots
Pretreatment with K (Pt)	667·21	275·1
Simultaneous treatment with K (S)	278·29	5·6
Rubidium (Rb)	611·04	36·7
Pt × S	2·17	5·5
Pt × Rb	8·44	17·6
S × Rb	219·00	6·3
5 % point	4·54	
1 % point	8·68	
0·1 % point	16·59	

† Data transformed to logarithmic basis for analyses.

No detailed interpretation of the interaction between rubidium and potassium with respect to the absorption and distribution of the former ion can yet be advanced. It is, however, apparent that the two ions interacted independently with respect to entry into roots and to transference across the cytoplasm to the stele. This situation is most simply interpreted by the postulate that the ions interact differently in exchange processes following their initial entry into the root, and in the subsequent process when they are transferred across the cytoplasm. Clearly, then, no conclusions with regard to the competition of ions for sites on the carrier can be reached on the basis of observation of changes in the absorption of one ion induced by varying the concentration of other components of the external solution.

Table 6. *Effect of* 2×10^{-2}M *potassium on the absorption of rubidium from solutions by young barley plants*

Duration of treatment (hr.) ...	4		20		
Concentration of rubidium in external solution (M × 10^{-3}) ...	1	10	1	5	10
Rubidium absorbed (mg./plant):					
K absent	0·050	0·079	0·234	0·320	0·385
K present	0·0078	0·071	0·031	0·126	0·233
S.D.	0·01		0·027		
% of absorbed rubidium present in shoots:					
K absent	19·5	27·1	64·0	67·3	68·0
K present	32·1	38·1	68·8	72·4	72·8
S.D.	5·25		1·6		

V. CONCLUSIONS

In the absence of any direct evidence as to the nature of the postulated carrier in active accumulation, no detailed interpretation can be possible of the mechanism whereby ions are transferred from the outer medium against an apparent concentration gradient to the vacuole or vascular stele. The evidence here presented indicates, however, that two distinct phases must be envisaged, namely, the entry of ions into the cytoplasm by exchange and the subsequent reaction involving the carrier already postulated. Discussion will here be confined to these two processes, but it is obvious that they do not embrace all reactions in which the entering ion may take part; the utilization of ions in metabolic processes near their site of entry may, as has been shown in the studies with phosphate, have a profound effect on the rate of absorption. The examination of the nature of these processes is beyond the scope of the present discussion, though the work of Kamen & Spiegelmann (1948) encourages the view that the phosphate metabolically retained in roots was involved in esterification processes.

Exchange mechanisms have frequently been described as *passive* or *physical* (e.g. Broyer, 1951) to distinguish them from the subsequent *active* step of accumulation. This terminology seems misleading, since ionic exchange or Donnan equilibria depend on metabolically produced substances equally with the active mechanism of accumulation. The apparent independence of respiration frequently shown by the passive process clearly indicates not independence of metabolism, but that metabolism is maintaining the process at a relatively steady state. This is readily demonstrated by the effect of gross changes in metabolism: sufficient concentrations of inhibitors destroy the exchange capacity of cells. Conversely, as Eddy & Hinshelwood (1951) have shown, the induction of a state of rapid glycolysis in resting bacteria causes the rapid entry of cations, apparently by an exchange process. Such effects are clearly of an entirely different category from those which lead to the transference of ions to cell vacuoles or to the vascular stele of roots. The total exchange capacity of the cytoplasm and probably also the affinity of different sites for ions must thus be expected to be variable, depending on metabolic activity. The variable effects of potassium on the transference of rubidium to shoots here described would appear to reflect such differences.

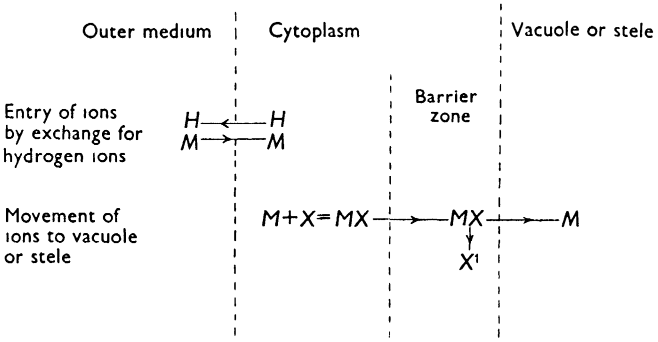

Fig. 8. Postulated mechanism for the metabolic accumulation of cations. (A similar mechanism for anions is envisaged.) M = entering cation. X = carrier produced by metabolism. X^1 = product of breakdown of carrier-ion complex.

A generalized scheme embracing the two processes, whereby it is suggested ions are metabolically accumulated, is proposed with more than a little trepidation in Fig. 8. For reasons stated earlier it is assumed that the influx of ions into the vacuole of single cells and into the vascular tissues of roots depends on broadly similar processes. However, it is to be expected that more detailed studies will reveal differences between the two mechanisms.

The outer layers of the cytoplasm are regarded as being relatively permeable to ions by exchange or diffusion. This conclusion is indicated not only by the marked isotopic exchange of cations which Broyer & Overstreet (1951) and Sutcliffe have demonstrated, but also by the ease with which phosphate which has been retained in metabolic processes in the roots subsequently diffuses into the outer medium. Both because of the marked concentration gradient which exists between the vacuole of cells and the outer medium and because of the apparently low exchangeability of ions which have penetrated sufficiently deeply into the cell (cf. Sutcliffe), the inner layer of the cytoplasm, presumably the tonoplast, must be regarded as a 'barrier' to the free movement of ions by exchange or diffusion. The transference of ions across this zone is considered to be effected by the carrier mechanism.

It is visualized that respiration leads to the production of a substance (X) which is freely diffusible throughout the cytoplasm, and which places ions under restraint on the outer side of the 'barrier', forming a complex which diffuses across the 'barrier' and breaks down to liberate the ions on the inner side. This scheme differs in no great respect from that put forward by Wohl & James (1942). Rosenberg (1948) has discussed the thermodynamic aspects of mechanisms of this type, and their existence has been suggested by Steward & Street (1947), and Jacobson *et al.* (1950). The view that the carrier may be amphoteric is encouraged by the fact that the present work and that of Sutcliffe indicates the existence of comparable mechanisms for cations and anions respectively.

While no direct information with regard to the nature of the carrier is available, the correlation between protein synthesis and salt accumulation which occurs in many tissues has led to the suggestion by Steward & Street that ions combine with nitrogenous compounds and are released when these compounds break down. Some evidence compatible with this view has been obtained in the present investigations; over periods of 3 hr. or longer, the absorption of phosphate by barley plants has been increased by the presence of nitrate (Table 7). It appears also that the absorption of rubidium is increased to a smaller extent in experimental periods of 24 hr.

Table 7. *Effect of* 3×10^{-4}M *nitrate on the absorption of phosphate by barley plants at the second leaf stage from a solution containing* 3×10^{-8}M-H_2PO_4

Duration of experiment (hr.)	Nitrate present	Nitrate absent	S.D.
3	1·63	0·96	0·27
6	2·01	1·42	
24	2·42	1·52	

An alternative interpretation of these results is, however, that the increase in respiration rate occasioned by nitrogen metabolism and not the production of nitrogenous compounds is effective in increasing absorption.

The energy relations of the carrier mechanism may now be considered. Since the earlier observations of Steward (1933) it has been apparent that the accumulation of ions is dependent on the release of energy by respiration. That two metallo-terminal oxidases, cytochrome and ascorbic acid oxidases, can effect the necessary electron transfer is now established and it appears from the work of Harley that flavoproteins may have the same capacity. A question of obvious interest is whether the formation of the carrier is the only step in the accumulation process which is quantitatively dependent on respiration. Some evidence that this is the case is provided by the fact that the carrier produced by prior respiration can apparently mediate the transfer of phosphate when respiration is subsequently inhibited. Results presented by Sutcliffe also support this suggestion. He considers it probable that metabolic energy is not involved in the combination of the ion with the carrier because this reaction can apparently occur at low temperatures. Moreover, the view that the breakdown of the carrier-ion complex is likewise independent of respiration is compatible with his observations of the effect of temperature changes between 15 and 30° C. on the absorption of cations. Increasing temperature within this range markedly accelerated the rate of absorption of ions, but the concentration in the tissues when equilibrium had been attained with the external solution was unaffected. The increased rate of transport when temperature was raised can be attributed to the effect of the increased rate of respiration on the rate of formation of the carrier. If, however, the association of the ion with the carrier and the breakdown of the resultant complex are not directly dependent on respiration, the equilibrium concentration in the cell will be determined by other factors; accumulation will cease when the slow outward diffusion of ions from the vacuole into the cytoplasm equals their rate of entry into the cytoplasm from the outer medium. This will result in the concentration of MX being constant throughout the system and its inward diffusion will therefore cease. Since both the diffusion of ions into the vacuole and the initial process of entry are expected to be little affected by temperature, the equilibrating concentration will be unaffected by metabolism. If, on the other hand, the breakdown of MX at the interior did depend on a glycolytic process, its rate would increase with rising temperature, and the equilibrium concentration in the tissues would be consequently affected. This accords with the conclusion of Lundegårdh (1950) that the release of ions into the stele is not linked with glycolysis.

The type of mechanism here discussed is unsupported by direct evidence. No published data appear, however, to be irreconcilable with it. The close correlation between the activity of cytochrome and salt absorption which has been demonstrated in great detail by Lundegårdh (1945, 1950, 1952, 1953*a*, *b*) cannot be regarded as proof of the theory of anion respiration. Such results can equally be interpreted as indicating that, in the tissues investigated, a carrier is produced by respiration through the cytochrome system, and that the rate of utilization of the carrier is an important factor determining the rate of its production. Similarly, the quantitative relationship between respiration and salt uptake demonstrated by Robertson & Wilkins (1948) can be interpreted as indicating that the transfer of one electron is required for the production of the carrier necessary to bring about the accumulation of one univalent ion of each sign. The difficulties Robertson, Wilkins & Weeks (1951) and Lundegårdh (1952) have experienced in reconciling the fact that 2, 4-dinitrophenol may inhibit salt uptake without inhibiting respiration can be explained by the postulate that this substance inhibits the formation of the carrier by interference with energy transfer.

VI. SUMMARY

Evidence is presented which indicates that respiration through the ascorbic acid oxidase system can mediate salt absorption in the roots of young barley plants. An examination of the effects of respiratory inhibitors on the absorption of phosphate by barley plants from dilute solutions leads to the conclusion that tissues can store the capacity to accumulate ions. It is therefore considered that the accumulation is affected by a carrier substance produced by respiration. The relationship between the transfer of ions across the cytoplasm and ionic exchange process is considered. The view is advanced that the accumulation of ions must be interpreted in terms of both exchange reactions and of the association of ions with a metabolically produced carrier.

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SALT ACCUMULATION IN PLANTS: A RECONSIDERATION OF THE ROLE OF GROWTH AND METABOLISM

A. SALT ACCUMULATION AS A CELLULAR PHENOMENON

By F. C. STEWARD and F. K. MILLAR

B. SALT ACCUMULATION IN THE PLANT BODY

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The problem of salt accumulation in plants necessarily begins with the process in single cells. This phase of the problem will be considered in Part A of this review.

With respect to a vascular plant the problem involves other considerations which lead to an understanding of what may be called the 'internal nutrition' of the organism and to the recognition in the vascular plant body of 'centres of growth and salt accumulation'. While such centres clearly involve the method by which individual cells accumulate solutes they also involve other interrelationships, and the problem thus impinges upon the larger ones of growth, development and morphology of the entire organism. An attempt will be made to analyse these questions in Part B of this review.

No attempt will be made to deal comprehensively with the extensive literature in this field; this will permit the presentation of some new data, and more particularly will allow space to develop certain speculative ideas.

A. SALT ACCUMULATION AS A CELLULAR PHENOMENON

I. INTRODUCTION

A principal feature of the *Nitella* experiments was the exchange of entering bromide for issuing chloride. It was tacitly assumed that this was a true exchange of bromide for chloride. However, in the experiments a large population of *Nitella* cells was used and the possibility, in fact the probability, existed that the bromide entered the younger and still growing, expanding cells, while the chloride issued from older and more senescent

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* Using data from experiments with S. M. Caplin, J. A. Harrison, F. K. Millar, R. Overstreet, B. M. Pollock, and A. G. Steward.

cells. An oft-quoted experiment showed that a given population of *Nitella* cells only attained a steady state with respect to the concentration of bromide and chloride in the cells after the lapse of a relatively long period, namely, 40 days. It is inconceivable that any biological process involving a large population of cells and requiring the lapse of so long a time should proceed independently of the concomitant changes due to growth and development. Therefore, even in the *Nitella* experiments, there was already some evidence that the actual development of the cells in question might be involved, in a determining way, in the intake of salt into their cell sap.

It was the experiments on storage tissue, particularly those on thin slices of potato tissue made in the first instance in Hoagland's laboratory (Steward, 1932*a*), that turned our attention and also that of Hoagland to the importance of respiration as the metabolic process that mediated the energy required by salt accumulation.

On previous occasions the work on salt accumulation, largely using cut disks of plant storage tissues as experimental material, has been summarized from the following points of view:

(*a*) From the standpoint of the role of respiration and metabolism in salt accumulation (Steward, 1935, 1937), recognizing that the respiratory and metabolic activity of the cell is involved because it furnishes the ultimate source of energy for the process, and that the relationship is more indirect than if the entry of ions was determined by the exit of a specified amount of carbon dioxide.

(*b*) From the standpoint of the status of the cell or organ for further growth and development, recognizing that their potentialities for growth, by division or cell enlargement, profoundly modify the way that metabolism may be used in salt accumulation (Steward, 1935) and that their previous nutritional status, i.e. whether they are of high or low salt content, will profoundly alter the amount of salt that may be absorbed (Hoagland & Broyer, 1936).

(*c*) From the standpoint of the varied metabolic processes that may be observed in potato cells that are accumulating salts (Steward & Street, 1947), recognizing that in order to understand this system one needs to know how these various processes (respiration, protein synthesis, oxidase activity, starch$\rightleftharpoons$sugar equilibria, etc.) are influenced by the principal variables that determine salt accumulation (the nature and concentration of the salt, oxygen tension, temperature, surface/volume relations of the tissue, the time drift after cutting the tissue, etc.).

From these various summaries and the papers to which they refer, the following main ideas and conclusions were obtained prior to the work now to be presented:

(i) In thin disks of potato tuber, intake of cation and anion from very dilute solutions may proceed in approximately equivalent amounts at rates which may be linear with time for long periods. The rates of ion intake are determined by oxygen tension, i.e. pO_2 in the gas stream (Steward, 1933) and temperature (Steward, Berry, Preston & Ramamurti, 1943).

(ii) The relative absorption (accumulation ratio) of bromide was greater from more dilute solutions. In very dilute stirred solutions, furnished in large volume, the accumulation ratio could be very high (Steward, 1932*a*). In the range 0·045, 0·0045, 0·00045 M-KBr a tenfold increase of external concentration doubled the internal concentration and the effect on the respiration during the period was small (Steward, 1933).

(iii) The effects of oxygen pressure on salt intake and respiration indicated that salt intake varied along with a large component of the aerobic respiration that was only oxygen saturated in solutions in equilibrium with air, and this component was probably mediated by the polyphenol oxidase system, rather than by a cytochrome system. The pO_2 at which oxygen saturation occurred was different for tuber tissue and for excised roots (Steward, Berry & Broyer, 1936).

(iv) The relation of salt intake to respiration did not only concern a part of the respiration due solely to the presence of the salt, but it varied with respiration and metabolism which occurred even in the absence of the salt and was determined by other variables (pO_2; temperature, time, proximity to the surface of the disk, etc.). The relation of respiration to salt intake was not regarded as a simple and direct one, nor was it the direct consequence of carbon dioxide production *per se.*

(v) Salt accumulation and the metabolism with which it is associated proceed at a greater intensity in the surface layers of cut disks, and it is in these cells that visible signs of growth and vital activity may be seen, especially in disks that are exposed to moist air.

(vi) To accumulate bromide within, potato disks require other properties than a high rate of respiration. These are the ability of the cells to grow and divide and to synthesize protein, properties which are eliminated after long storage of tubers at low temperature (Steward *et al.* 1943). In the case of artichoke tuber (Steward & Berry, 1934) the intake of bromide is linked with properties which change with the lapse of time after cutting, absorption being higher at first and declining thereafter.

(vii) The variations in the metabolism brought about by the nature and concentration of the external salt all tend to show that at least a major part of the aerobic respiration of the tissue slice is mediated in ways in which protein synthesis and respiration vary together. Parallel effects due to salts on respiration and protein synthesis were observed, and these were ascribed primarily to the cations, with contrasting K : Ca effects, but were modified by the anions. Throughout, the effects of salts and oxygen on respiration and on the use of soluble nitrogen compounds in protein synthesis run parallel (Steward & Preston, 1940, 1941*a*).

(viii) External pH's of 7·0 promoted respiration and protein synthesis, but sharply contrasted effects were observed at constant pH due to the use of CO_2/HCO_3^- or phosphate buffers. Whereas increased phosphate concentration increased respiration and protein synthesis, increased concentrations of the CO_2-bicarbonate buffer at pH 7·0 decreased it and could eliminate bromide accumulation altogether (Steward & Preston, 1941*b*).

(ix) In thin slices of storage tissue many vital processes are linked together. Increased respiration, the activity of the oxidase system, synthesis of protein from soluble nitrogen compounds and salt accumulation all tended to run parallel as affected by external conditions. Protoplasmic streaming (Steward, 1932*a*) was also a visible indication that while the tissue was doing osmotic work in accumulating salt it was also capable of mechanical work. But the property that above all expresses the ability of the cells to accumulate salts seemed to be their ability to synthesize protein—if this were lacking much metabolic acitivity and respiratory carbon dioxide seemed to be of no avail.

(x) The effects of rapid aeration were now seen to be twofold. First it furnishes oxygen at the appropriate pressure for respiration and salt accumulation. Secondly, it sweeps out carbon dioxide as fast as it is formed. In the latter way carboxylation reactions are limited and decarboxylations are fostered (Steward & Street, 1947). Thus the high rate of respiration that obtains under these conditions is maintained largely, if not solely, by use of the carbon residues from deaminated amino-acids—residues that can be fed in to the Krebs cycle in lieu of the breakdown products from sugar. Meanwhile the nitrogen groups thus transferred form protein, using a carbon framework derived from sugar. It is this 'nitrogen cycle' that is fostered by rapid aeration and is inhibited by carbon dioxide. In 'handing on' the nitrogen for protein synthesis, glutamine and glutamic acid play the key role. Any treatment which retards, or stops, this synthesis of protein, retards or eliminates the accumulation process (cf. effects of CO_2/HCO_3^-, $[Ca^{++}]$, prior storage at low temperature).

(xi) In mediating the salt intake the role of protein synthesis was conceived to involve a carrier molecule. If this molecule (say a phosphorylated glutamine) could bind (or hold) anions and cations, transport them across the cytoplasm, then, if it were condensed to protein, it might leave the ions in a situation in which they might be readily accumulated in the vacuole (Steward & Street, 1947, p. 496; cf. Franck & Meyer, 1947).

(xii) The scheme (Steward, 1935) which attempted to relate the activity of ion-absorbing systems to their growth and development reached the following conclusion. If it were possible to deal with cells that were actively dividing and multiplying, as in a meristem, the *concentrations* of the solutions they contained would be greater than at any other time, although the *amounts* per cell would be limited by the small volume of the aqueous phases in strictly dividing cells. This seemingly pardonable extrapolation of the data then available was based on such facts as that approaching the root apex the concentration of ions absorbed in the total water of the cells steadily increased. Also bromide is accumulated in potato cells as they reapproach the conditions in which cell division may occur and, if the cells are treated in such a way that the power of division is eliminated, the power of accumulation disappears also. Slices of potato tissue in which protein breakdown occurs even lose their solutes to an external solution.

In this state of affairs a new approach to the problem was required. Before describing this, reference should be made to the work of Sutcliffe

(1952) since this followed the general pattern of the work summarized above.

After exposing beetroot disks to three changes of distilled water at hourly intervals they were treated with daily changes for periods up to 8 days. In consequence the K content of the tissue declined to half its original value. On subsequent transfer to relatively strong solutions (up to 0·04 M-KCl) K was reabsorbed in such a manner as to suggest

(i) The initial rate of uptake was a function of the salt and solute deficit created by the long washing.

(ii) The final plateau of K content attained after many days was relatively unaffected by either the concentration of the KCl or the initial deficit due to the long washing but was in fact the maximum that these cells could contain.

(iii) This intake of K, clearly induced by the long treatment, was cyanide inhibited and the tissue acquired a more conspicuous salt-induced, cyanide sensitive, component of respiration the longer it was pretreated with distilled water. There was, however, no simple relation between K absorbed and salt-induced respiration. (The respiration of beet tissue in water was surprisingly sensitive to cyanide.)

The fundamental feature of these experiments, however, still evades us. What were the metabolic consequences of the long washing with distilled water (cf. Steward & Preston, for the potato tuber, 1940)? What happened to all the other cations and the various organic electrolytes and non-electrolytes (cf. Steward, 1932*a*)? Is it conceivable that, without obvious growth, the tissue would increase its total K content threefold over its initial value unless:

(*a*) The pretreatment depleted the cells of salts and solutes.

(*b*) Placing the tissue in such a strong solution (0·04 M-KCl) permitted it to embark upon a vicarious intake of salt, unaccompanied by growth, till the cells readjust to this applied concentration. This is an effect comparable to that induced in Hoagland's 'low salt' roots though here it is due to a superimposed high concentration of salt.

(*c*) With the elapse of time the metabolic characteristics of the cells changed in response to aeration. It is still possible, lacking evidence, that these changes were in the direction of growth (e.g. protein synthesis) even though they did not proceed to actual cell division or proliferation.

The objective of the new approach was to find the point of contact in the metabolism of growing cells between the diverse processes of water and salt intake on the one hand and respiration and protein synthesis on the other. Two main requirements seemed to be apparent.

First, biochemical techniques were required by which to fractionate the nitrogen compounds in the hope that the intermediary metabolism involved in the protein synthesis might be traced. The technique that had emerged and was adaptable to this end was the qualitative and quantitative technique of paper chromatography (Steward & Thompson, 1950; Thompson, Zacharius & Steward, 1951; Thompson & Steward, 1951). In this way, the nitrogen compounds of the potato-tuber tissue have been described and some investigations have been made of the changes which occur under conditions conducive to salt intake (Steward & Thompson, 1950).

The second requirement was quite different, for one needed a system in which cells could be placed at will in either of two contrasted states, namely, the proliferating-dividing condition or the non-dividing condition.

The search for such a tissue system prompted a re-examination of tissue-culture techniques. Again the detour proved rewarding beyond expectations, but, from the standpoint of this review, the desired system was found in the use of standard explants of secondary phloem from the carrot root. Under the conditions described (Caplin & Steward, 1948, 1949) such explants will either remain under sterile conditions with only sluggish expansion for a long period of time, or, if supplied with nutrients, vitamins and the growth factors that are contained in coconut milk, they will embark on a most remarkable period of active growth by cell division.* In the coconut-milk factor, or factors, lay the secret of the transition from the relatively inactive non-growing state to the actively proliferating, growing condition. Here then was the type of system desired for a study of the kind of salt absorption which is characteristic of the cells in these two states and which would enable one to recognize the metabolic features which, in these cells, are linked with salt accumulation. The mechanical devices by which these ends were achieved have been described and may, therefore, be merely referred to here (Steward, Caplin & Millar, 1952).

II. ABSORPTION OF ^{137}Cs BY GROWING AND NON-GROWING TISSUE CULTURES

Here a confession is necessary. The first expectation was that the actively growing, proliferating carrot-tissue cultures, which increase in fresh weight some eighty times in 20 days, would, if supplied with an ion that could be absorbed, absorb much more actively than their relatively non-growing analogues whose weight, though maintained, was only increased slightly in comparison. By the use of the radioactive isotope ^{137}Cs it was possible to investigate this question quite accurately, and this contribution is especially associated with Miss Millar (1953). The unexpected and dramatic result was as follows: per unit weight the relatively non-dividing cells of the carrot explant absorbed *more* caesium than the dividing cells of the growing cultures (see Table 1). This result was too striking to be ignored.

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* Using maceration techniques that he has applied to the interpretation of the growth of roots, Dr R. Brown, working with one of us (F.C.S.), has examined these carrot cultures with the following results. The initial tissue explant weighing 2·6 g. contained about 25,000 cells. After about 13 days the number was over 1×10^6 if the explant was in the medium containing coconut milk under the standard conditions referred to above.

Table 1. *Fresh weight and absorption of* ^{137}Cs *by proliferating and non-proliferating carrot explants in carrier-free solution*

Days after inoculation ...	0	2	4	6	10	14
*Growing cultures, mg. fresh weight	4·0	5·7	7·3	11·5	31·8	87·0
Counts/sec./mg. fresh weight	—	1·14	2·17	2·85	2·55	2·14
†Non-growing cultures, mg. fresh weight	4·0	5·1	5·9	6·3	7·2	8·4
Counts/sec./mg. fresh weight	—	2·37	4·87	8·17	17·0	30·4

* Rapidly proliferating cultures in basal medium + coconut milk.

† Cultures which only very sluggishly expand in basal medium only without coconut milk.

The ^{137}Cs could be obtained and used in carrier-free solution (10^{-8} mol./l.), and by addition of non-radioactive caesium (at 10^{-3} mol./l.) one could vary the total caesium concentration over a very wide range ($\times 10^5$), keeping the concentration of radioactive caesium constant. Again it was found that the relationship of these two types of absorbing system, the growing and the non-growing, to the total concentration of caesium were quite different. The growing-dividing cells behaved again in a quite unexpected fashion (Table 2).

Table 2. ^{137}Cs *absorption ratio* as a function of time for proliferating and non-proliferating carrot-tissue cultures with and without the addition of inert carrier caesium*

Days after inoculation ...	4	7	10	14
†Growing cultures with carrier caesium absorption ratio	7·30	9·14	14·1	13·4
Growing cultures without carrier caesium, absorption ratio	9·43	10·7	12·9	14·5
‡Non-growing cultures with carrier caesium	27·2	53·1	77·7	79·5
Non-growing cultures without carrier caesium	375	649	2050	2130

* Absorption ratio is counts/sec./g. fresh weight of tissue divided by counts/sec./c.c. of external solution.

† Rapidly proliferating cultures in basal medium + coconut milk.

‡ Cultures lacking coconut milk.

Table 2 gives the data for uptake of ^{137}Cs in terms of its absorption ratio, i.e. the concentration of ^{137}Cs in the total water of the tissue divided by the final concentration of ^{137}Cs in the external solution. From the data in Table 2 the surprising result was that the presence of a relatively large excess of caesium ions over the ^{137}Cs had very little, if any, significant effect on the absorption of the ^{137}Cs in the case of the growing-proliferating cultures.

The results could only be interpreted on the very definite conclusion that the caesium absorbed by growing-proliferating tissue cultures *bore a linear relationship to the external concentration, that is, to the first power of the external concentration.* Because that external concentration was varied over such a wide range (× 10^5) this result was most surprising.

In contrast the relatively non-growing tissue cultures, that is, those lacking the coconut milk growth factor(s), and merely placed in a calcium chloride solution, behaved in a more conventional fashion because the relative uptake of ^{137}Cs was greater from the more dilute solution. (In other words, the accumulation ratio increased with dilution of caesium in the familiar fashion.)

These results lead to the following conclusion. In the angiosperm plant body the complete cycle of events from cell division, through the formation of the vacuole and the complete enlargement to the mature cell, is characterized by salt absorption which proceeds at two distinct stages by two distinct types of mechanism.

The first of these (stage I) is characterized by, or is emphasized in, the cell in the state in which it is capable of continuous and active cell division, while it remains small; in this state the cell absorbs its ions in such a way as to suggest that they are bound on certain sites produced continuously and in the process of this growth. The relationships to concentration clearly suggest that this binding is a stoichiometrical one, the suggestion being that positive ions like caesium are bound to negative, or acidic, locations. Such a process would be expected to reside specifically in the cytoplasm where the synthesis occurs.*

The second type of absorption mechanism (stage II), however, is quite different, and it is characteristic, not of the dividing cell, but of the cell in which division has slowed down and expansion of the vacuole is the chief event. This type of absorption, now regarded as a process of secretion into the vacuole, is characterized by the familiar accumulation mechanism which causes the relative absorption to be greatest from the most dilute external solution. Both of these absorption processes are related to time and aeration in ways that necessitate that the metabolism of the cells determines the intake of the ion.

However, we can now see that the extrapolation in the earlier scheme of 1935 to the state of active cell division was not wholly justified. At the point where growth ceases to be predominantly by cell division and becomes predominantly by expansion, an abrupt change occurs in the *nature* of the

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* Even in the 'bound' state the ion may be 'accumulated' in the sense that the amount present, expressed as a concentration in the total water of the cell, may be much greater than in the external solution. Ratios of 8 to 10 were observed in these experiments.

absorption process. It is difficult to see how this important conclusion could have been reached without the particular advantages presented by the tissue-culture system to which reference has been made. The obvious suggestion is that with the formation, or rather the enlargement, of vacuoles the activity of the tonoplast intervenes, in the way that de Vries originally visualized (1885), to cause secretion internally into the vacuole.

One may now ask what are the other metabolic characteristics of the cells in these two contrasted states.

The dramatic metabolic effect produced by the coconut-milk growth factor on the carrot-tissue cultures is the marked stimulation to protein synthesis which accompanies the cell division. Cells of the resting carrot, like those of potato tuber, contain a relatively large percentage ($>50\%$) of their total nitrogen in the form of soluble nitrogen compounds.* In the cells cultured on coconut-milk media this is not so, for the bulk (70%) of the nitrogen is now present in the protein form (Steward *et al.* 1952). Much more striking, however, is the fact that the proportion of the different substances in the alcohol-soluble nitrogen fraction is quite different in the growing culture and in the relatively non-growing culture. This is particularly true of the amide, asparagine, which is conspicuous in the non-growing cells and is either absent, or is very much reduced, in the growing cells.

The same sort of result has been demonstrated by the use of small explants of potato tissue which, however, require the intervention not only of coconut milk but of 2, 4-D, or an analogous substance, in dilute solution to make them grow (Caplin & Steward, 1951). One can, therefore, relate the first kind of ion absorption which occurs in growing-dividing cells and which is proportional to the first power of the external concentration of the absorbed ion, to cells in a particularly active state of protein synthesis and cell division and, therefore, of multiplication of their self-duplicating units. Accompanying the transfer from the resting to the dividing state the composition and metabolism of the tissue changes profoundly. Even the protein that is synthesized in the dividing cells is recognizably different, for it may be shown by quantitative paper chromatography that it contains hydroxyproline, unlike the protein of resting cells, and contains other amino-acids in somewhat different proportions. Moreover, it seems permissible to regard the binding sites which take up the ion in question as being formed continuously as the new protein is synthesized. So long as the coconut-milk factor is present the carrot cells are arrested in their

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* The data on nitrogen metabolism of tissue cultures were communicated by Steward & Thompson to the American Society of Plant Physiologists at Minnesota, September 1951, and they will appear in the *J. Exp. Bot.*

differentiation in the sense that they continue to divide but they do not fully enlarge and vacuolate, nor do they pass to the state of maturity in which cell divisions would not occur.

Therefore, what the carrot-coconut milk system does is to enable one to separate the two steps (stages I and II) of the absorption process giving emphasis, at will, to the kind of absorption which is characteristic of the growing-dividing plant cells or to that which is characteristic of the cells whose further growth is mainly by enlargement, rather than by division.

Metabolic inhibitors have been used to characterize even further the differences between cells in these two contrasted states (Steward & Shantz, 1951). The effect of various inhibitors upon the growth of carrot cells in coconut-milk media is shown in Table 3. The striking thing is that the growth of the carrot in presence of coconut milk is so *insensitive* to cyanide. From what is known of the effect of cyanide upon respiration this would seem to imply that much of the respiration proceeds over pathways which do not directly involve cytochrome, or other enzyme systems catalysed by heavy metals.

Table 3. *Effect of enzyme inhibitors on growth (mg. fresh weight per culture) of carrot-tissue explants* during* 21 *days in medium + coconut milk*

Inhibitor	Mean fresh weight (mg.) at specified conc. of inhibitor		
	10^{-3}M	10^{-4}M	10^{-5}M
Fluoride	132	141	161
Cyanide	147	134	117
Dinitrophenol	10	11	79
Dinitrocresol	4	5	13

* Weight of initial explants = 4·0 mg. Weight of explants 21 days in basal medium only = 7·0 mg. Weight of explants 21 days in basal medium + coconut milk = 137 mg.

The effects of a long (7 days) exposure to cyanide on both the non-growing cultures in calcium chloride solution and the growing cultures in basal medium + coconut milk are shown by its effect upon ^{137}Cs uptake. The data are shown in Table 4.

These data show a relatively greater sensitivity to cyanide in the case of the ion intake typical of the non-growing cultures than of that typical of the growing ones.

The effects of cyanide upon the respiration of the tissue in the two states have not yet been fully worked out. It is clear that the tissue which grows in media containing coconut milk, whose growth and ion intake are alike relatively insensitive to cyanide, produces carbon dioxide by pathways that are largely insensitive to cyanide. The tissue of the experiment in Table 4 which had been treated for 7 days with potassium cyanide when removed to a Warburg apparatus, produced carbon dioxide at the rate of 0·337 μl. O_2/mg./hr. while still in the presence of 10^{-4}M-KCN. Comparable tissue not treated with cyanide respired at the rate of 0·372 μl. O_2/mg./hr. Therefore, the respiration of the growing tissue is only suppressed by 10^{-4}M-CN to the extent of about 10%.

Table 4. *Uptake of* ^{137}Cs *by carrot tissue explants as affected by* 10^{-4}M-CN *during* 7 *days*

Type of culture	Medium	^{137}Cs uptake per day (counts/sec./mg.)		% Inhibition by CN
		CN-treated	Control	
Non-growing	$CaCl_2$	0·363	5·36	93·2
Growing	Basal medium + coconut milk	0·0225	0·0259	13·1

The respiration of normal carrot tissue as freshly excised from the carrot root is well known to be cyanide-sensitive (Marsh & Goddard, 1939). Our own data show that even after 2 days under the culture conditions described the respiration was inhibited to the extent of 40% by 10^{-4}M-KCN. Therefore, the ion intake which occurs in the non-dividing tissue is associated with a respiratory metabolism which is markedly cyanide-sensitive, particularly in freshly explanted tissue. Whereas the growing cell in the presence of coconut milk is relatively less sensitive to cyanide, as compared with the non-proliferating tissue in the absence of coconut milk, the converse is true in the case of those inhibitors that affect phosphorylation. Such enzyme inhibitors as the nitrocresols markedly inhibit the growth of the proliferating cultures, and their effect on ion intake will now be examined (Table 5).

The effects of treatment with nitrophenolic inhibitors were tested on cultures which grew in full nutrient + coconut milk for 21 days but which received the inhibitor at two stages, 0–2 and 7–9 days. The effect of the

Table 5. *Effect of dinitrocresol on growth and subsequent* ^{137}Cs *uptake by carrot-tissue cultures grown* 21 *days in presence of coconut milk*

All relative data for the inhibitor treated cultures expressed as a % of the untreated.

Conc. of inhibitor	Period of contact with inhibitor				Period of contact with inhibitor			
	0–2 days		% inhibition		7–9 days		% inhibition	
	Rel. F.W. (%)	Rel. ^{137}Cs uptake (%)	F.W.	^{137}Cs	Rel. F.W. (%)	Rel. ^{137}Cs uptake (%)	F.W.	^{137}Cs
10^{-4}	28·9	70	70	30	4·1	34·7	96	65
10^{-5}	93·5	106	—	—	81·1	90·2	—	—

inhibitor is recorded in terms of the total growth (mg. fresh weight) and the ^{137}Cs uptake which occurred subsequent to the treatment in comparison with the uninhibited controls. The data show that marked inhibition occurred as a result of contact with 10^{-4}M-dinitrocresol but that 10^{-5}M was much less effective. They also show that the inhibitor applied at 7–9 days reduced both the fresh weight and the ^{137}Cs uptake proportionally much more than when it was applied to the tissue at 0–2 days. (There is a lag period of approximately 4 days after inoculation, during which little or no external growth of carrot cultures in coconut milk occurs.) The relative insensitivity of the carrot cultures exposed to cyanide in the early period (0–2 days) is therefore to be associated with conditions in the tissue *before* it has fully responded to the coconut-milk stimulus.

Therefore, cyanide inhibition of metabolism and ion intake is typical of the freshly explanted tissue and of the non-proliferating tissue, i.e. tissue not treated with coconut milk. On the other hand, nitrophenolic inhibition of metabolism and of ion intake is typical of the cultured tissue in media that contain coconut milk. Furthermore, the effect of the nitrophenols on the tissue in coconut milk is most marked after time has elapsed and the tissue has fully responded to the coconut-milk treatment.

Both of these salt-absorbing mechanisms should be regarded as dependent upon oxidative metabolism. However, the first stage (stage I) of the absorption process, i.e. the one in which ionic binding is mainly in question, is closely linked to protein synthesis and to multiplication of cells and of their self-duplicating units. Although this mechanism is relatively cyanide-insensitive, phosphorylation seems to be necessary for its maintenance, since it is more sensitive to nitrophenols.

In the second type (stage II) of the salt-absorbing mechanism, where any concomitant protein synthesis is unaccompanied by cell proliferation, cyanide inactivates the mechanism. Here the prime event is the attainment of relatively high concentrations in the total water of preformed cells, and this involves the removal of the ions from their initial binding sites and their secretion into the vacuolar fluid.

III. ABSORPTION OF ^{137}Cs BY ARTICHOKE TISSUE: GROWING AND NON-GROWING

Proof of these two types of absorption process by reference to another tissue system was clearly desirable. Use was therefore made again of tissue from the Jerusalem artichoke tuber. In passing from its initial high rate of respiration through a time drift (when respiration falls to an eventually low level) cut disks of artichoke-tuber tissue pass from an active absorbing condition to a less active condition (Steward & Berry, 1934).

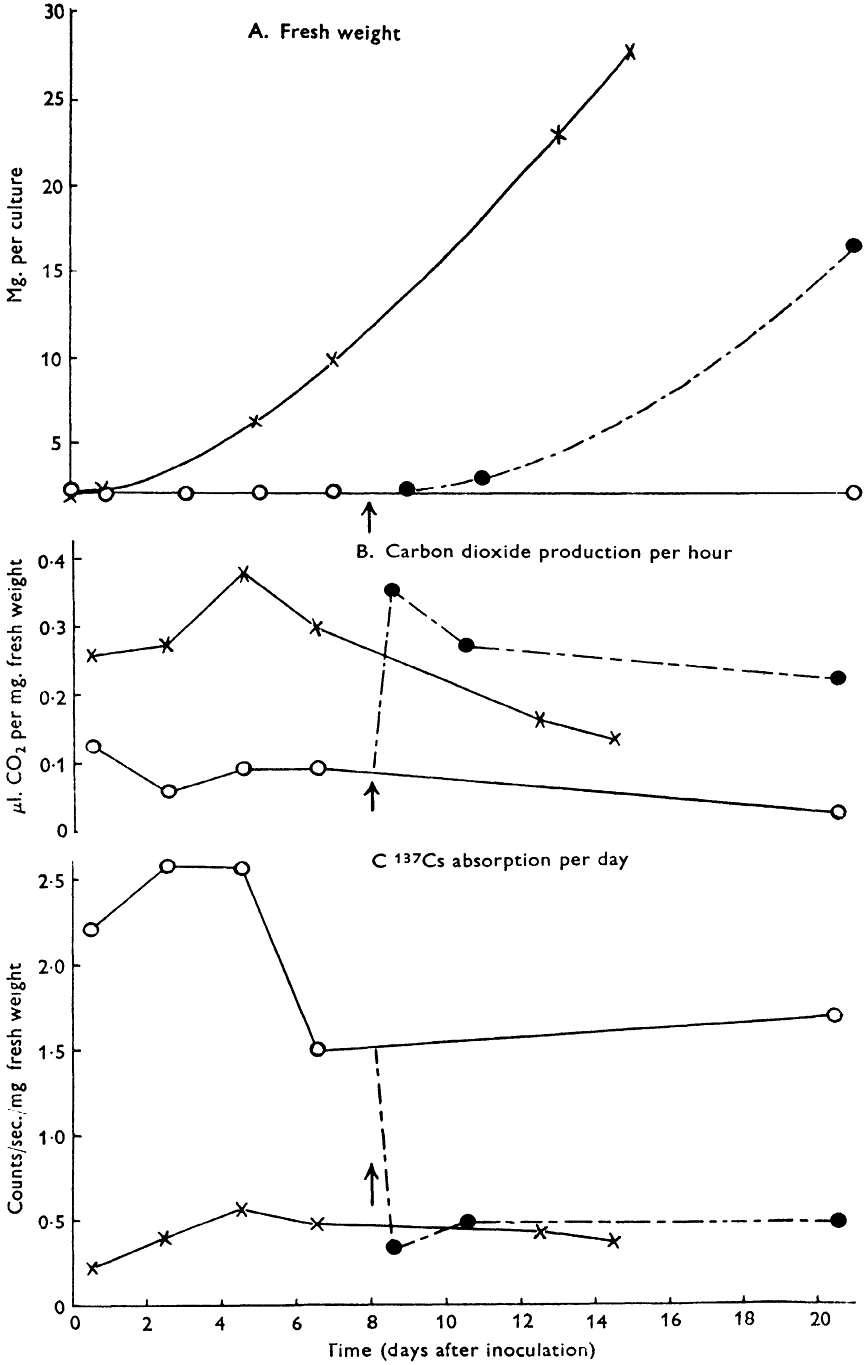
If coconut milk is added to artichoke tissues which, with the lapse of time and through exposure to solution, had become adjusted to a low rate of respiration (order of 0·08 mg. CO_2/g./hr. in contrast to 0·25 mg./g./hr. for fresh-cut disks), the cells (like those of the carrot) will return to the actively dividing state and will grow, and their rate of metabolism is increased. (Prior to our knowledge of this effect of coconut milk all attempts to raise the respiration of artichoke disks, which had passed through their time drift in distilled water, to their initial high level and to maintain this by the use of such added metabolites as phosphate, nitrate, sugar, amides, etc., had failed (Ramamurti, 1938). The effect of the coconut milk is, therefore, a highly specific one.)

One might have expected that the coconut milk and its attendant growth would cause the caesium uptake per unit weight of these cells markedly to rise. On the contrary, the tissue now takes up *less caesium per unit weight per unit time* than the controls which lacked the coconut-milk growth factor during the period of absorption. The data of Fig. 1 show that these effects attributable to coconut milk may be brought about at will in the artichoke tissue. This and other results confirm, for the case of the artichoke tissue, that the new ideas on salt intake which have been described apply also to these cells as well as to those of explanted carrot-root phloem.

The tissue lacking coconut milk absorbs ^{137}Cs in the manner of the growing but non-dividing cells (Text-fig. 1 C), its ion intake decreases during the time drift with the respiration in the manner previously referred to for bromide. However, by addition of coconut milk at points far along the time drift, e.g. 8 days, the cells change their metabolism, grow and, thereafter, absorb *less*, rather than more, ^{137}Cs per unit of fresh weight (Fig. 1 C). It should be noted that on passing from the non-dividing to the dividing state the tissue produces much *more* carbon dioxide but absorbs much *less* ^{137}Cs (cf. Text-fig. 1 B, C).

The comment may well be made that these experiments have not simplified the problem, they have only complicated it. Instead of one mechanism of salt intake and accumulation we now have two. And each of these mechanisms bears its characteristic relationship to the metabolism that is involved, particularly to protein synthesis. Also it may rightly be claimed that it is hardly a simplification to regard an explanation of salt intake as carrying with it the need also to explain something about the perhaps still more mysterious process of protein synthesis.

Therefore, a further digression must be made, but this will be justified because it leads to a possible picture of the events that may underlie the kind of result that has been described.



Text-fig. 1. Effects of time and coconut milk on fresh weight, carbon dioxide production and ^{137}Cs absorption of artichoke-tuber tissue cultures. ×——× cultures in basal medium + coconut milk; ○——○ cultures in calcium chloride; ●——● cultures transferred at 8 days (↑) from calcium chloride to basal medium + coconut milk.

IV. PROTEIN SYNTHESIS AND ION BINDING: A TEMPLATE HYPOTHESIS

The task now to be attempted is as follows: Some picture should be formed of how protein synthesis may occur at the expense of the soluble nitrogen compounds. This picture should visualize the central role which glutamic acid may play as the starting-point from which a variety of amino-acids may be derived by transamination. It should also give prominence to the role which the amide glutamine seems to play, for it is related to protein synthesis in ways that suggest that it is peculiarly able to donate nitrogen to the protein synthesizing surface, so that one may visualize the soluble nitrogen as being canalized through the form of glutamine. We should also be able to visualize how, by the aid of respiration, *both* protein synthesis and ion intake are enabled to proceed so that the nitrogen for synthesis, the energy for synthesis and salt accumulation and the ions to be accumulated seem to be presented to the active surfaces simultaneously and in a compatible form.

The possibility exists that the relation of protein synthesis to ion accumulation may concern the entropy changes. Formation of highly ordered large molecules from disordered amino-acid moieties will cause the kind of entropy change that might give a positive change of free energy if the accompanying heat change is small.

Dounce (1952) has outlined the template hypothesis in a detailed manner. He has visualized the chemical reactions by which a nucleic acid surface could reduplicate itself, and he has also visualized how it could permit the amino-acids to be combined with the template and finally removed again, or 'peeled off', in such a way that when they are removed protein is formed. First Dounce visualizes that the nucleic acid surface is activated by phosphorylation. By amino-transphorases the amino-acids are induced to combine with the nucleic acid surface. It is thought that when the protein is removed the adjacent amino-acids are induced to form the α-peptide links that exist in the protein. This is shown in Text-fig. 2.

Hanes, Hird & Isherwood (1950, 1952) and Hanes, Connell & Dixon (1952), however, have elaborated another idea. Glutamic acid and ammonia, with energy supplied from ATP, itself regenerated by respiration, can form glutamine (Speck, 1947; Elliott, 1948). Glutamine can be regarded as a hydrogen peptide in which the energy of respiration produces the amide linkage. Hanes has drawn attention to the fact that the single most important step in protein synthesis may well be the formation of the γ-glutamyl bond which can be built into a series of γ-glutamyl dipeptides. This process requires the intervention of systems which incorporate glutathione.* Enzymes exist in both plants and animals by which the glutamyl moiety of glutathione may be split off and combined with a variety of amino-acids to give γ-glutamyl dipeptides. The residue cysteinyl-glycine may then be available to regenerate glutathione, by using again the energy of respiration and by a process not dissimilar to that which has been described for glutamine. So the cycle could go around again.

The importance of this mechanism is that it visualizes a means by which a variety of γ-glutamyl dipeptides may be formed, and to do this one needs catalytic amounts of glutathione and the means, through respiration and phosphate band energy, to regenerate the glutathione from the cysteinyl-glycine residue and glutamic acid. However, the apparent dilemma is that the variety of peptides so formed do not incorporate the α-amino-peptide linkage which is actually necessary for the formation of protein. (The dilemma may of course be overcome by assuming that once energy is incorporated into these γ-dipeptide linkages the appropriate α-dipeptide link may be formed by some intramolecular rearrangement.)

Here, however, one can bring the ideas of Dounce and of Hanes together. If it is assumed that the amino-acids were presented to the template, consisting of a pentose nucleic acid surface, not in their free state, but only in the form of these γ-glutamyl peptides then some interesting consequences could follow. First, one could dispense with the need to phosphorylate the nucleic acid surface, because the energy inherent in the γ-glutamyl bond would suffice to enable the amino-acid to combine with the nucleic acid surface. The glutamyl residue would then become available for resynthesis to glutathione and so permit the reversible cycle to continue. By this means all the amino-acids could be presented in the form of the *same linkage*, the γ-glutamyl linkage, for which the nucleic acid surface could be regarded as a dipeptidase or transferase.† In fact, according to Brinkley (1952), the hydrolysis of peptides by nucleic acids has already been demonstrated, even though they are virtually free from a protein moiety. Since some synthetic resins are also now known to hydrolyse protein (Underwood & Deatherage, 1952*a*, *b*) this does not appear as improbable as it might otherwise have seemed to be. The remaining process of removing the amino-acid from its combination with the nucleic acid surface and, in the process, the formation of α-peptide links remains exactly as under the Dounce hypothesis.

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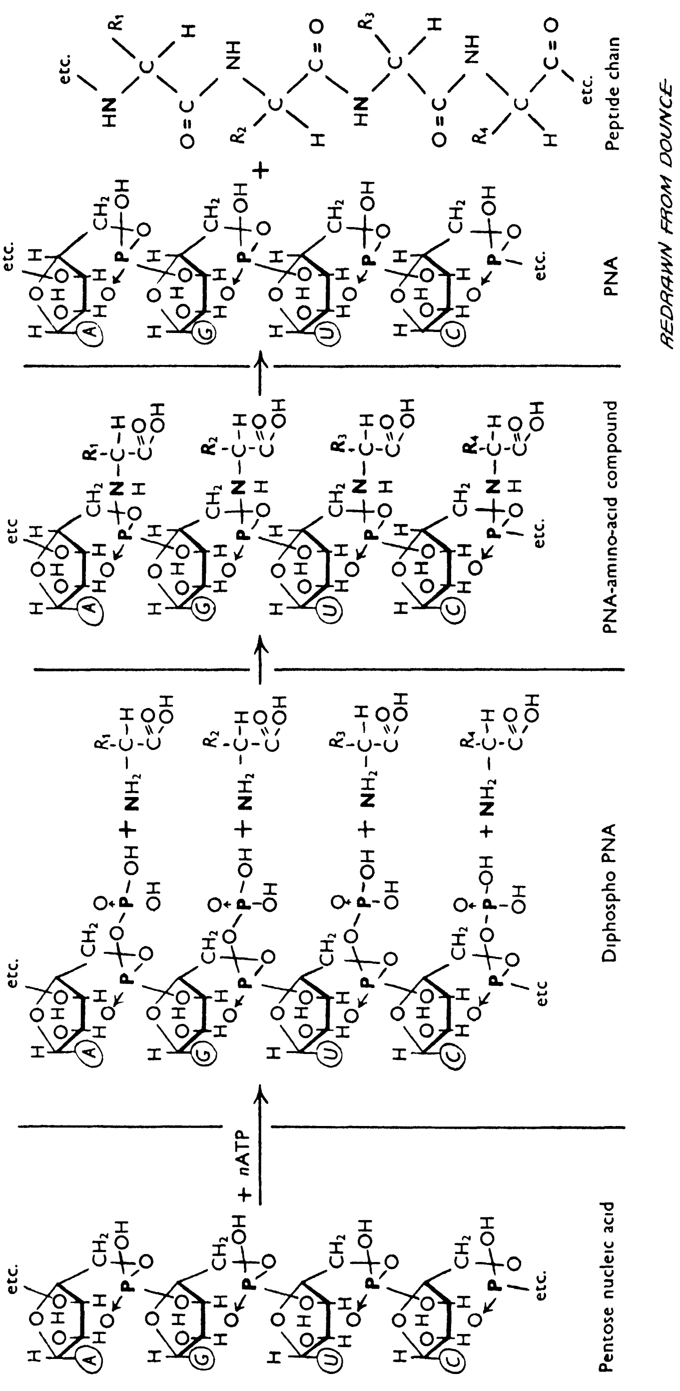
* Brinkley (1952) visualizes an activated substrate such as 'γ-glutamyl-coenzyme-A' as the actual donor of the glutamyl residue, so that glutamine and glutathione are reserves of this radical.

† It is true that the transfer in question, would be of the 'amine transfer' kind (Hanes *et al.* 1950), which though possible, is not yet demonstrable with isolated enzyme systems.


Pentose nucleic acid
+ nATP
Diphospho PNA
PNA-amino-acid compound
PNA
Peptide chain
A
G
U
C
etc.
REDRAWN FROM DOUNCE

Text-fig. 2.

Thus, one can now regard the role of glutamine and glutamic acid in protein synthesis in the following way. Glutamic acid is the acid which, more than any other, enables the amino groups to be handed on to other keto-acids through transamination, to generate, in the variety required, all the amino-acids necessary for protein synthesis. This would leave α-keto-glutaric acid, which would either be drawn into the Krebs cycle and respired away or regenerated to glutamic acid by nitrogen drawn from soluble nitrogen compounds that are not immediately available for protein synthesis. By the aid of respiratory energy, glutamic acid could form glutamine and by similar processes glutathione could also be produced. Through the sequence already described glutamyl peptides could be formed in almost any variety, so that glutamine would become the agent through which, in the necessary variety and proportions, the various amino-acids could be presented to the protein synthesizing surface and, in so doing, the peptide would bring up, not merely the amino-acid, but also the energy in the γ-glutamyl bond necessary to combine it with the nucleic acid template.

To relate these ideas to salt accumulation one needs to make still one more hypothesis. If instead of regarding glutamine itself, possibly phosphorylated, as the carrier molecule of ions (cf. Steward & Street, 1947), transporting them from the external surface to the place where protein synthesis occurs, one could now regard the γ-glutamyl-peptides as being the carriers, some interesting consequences follow.

Such peptides would have, or could have, free acidic groups that would bind cations and free amino groups which could bind anions. In the form of the complex the ions could be transported across the cytoplasm. If one regarded the seat of synthesis as in the vicinity of the tonoplast, then the ions with the amino-acids would be bound to the nucleic acid surface, as to an ion-binding resin when by the dipeptidase action, the amino-acid residue was fixed to the nucleic acid surface with the liberation of glutamine.

So long as new nucleic acid surfaces were being created, i.e. so long as self-duplicating surfaces are being multiplied, and so long as the amino-acid could be fixed and remain bound in this way, then no *free* accumulation in the vacuole would be expected to occur. This is visualized as the condition which obtains in the growing, dividing cell as exemplified by the cells stimulated to divide by coconut milk (stage I). Probably similar circumstances also obtain in meristematic cells proper.

However, when further synthesis of nucleic acid ceases, and if protein synthesis is to continue, the bound amino-acids should be ‘peeled off’ in the form of free protein. In doing so and at one and the same time, the

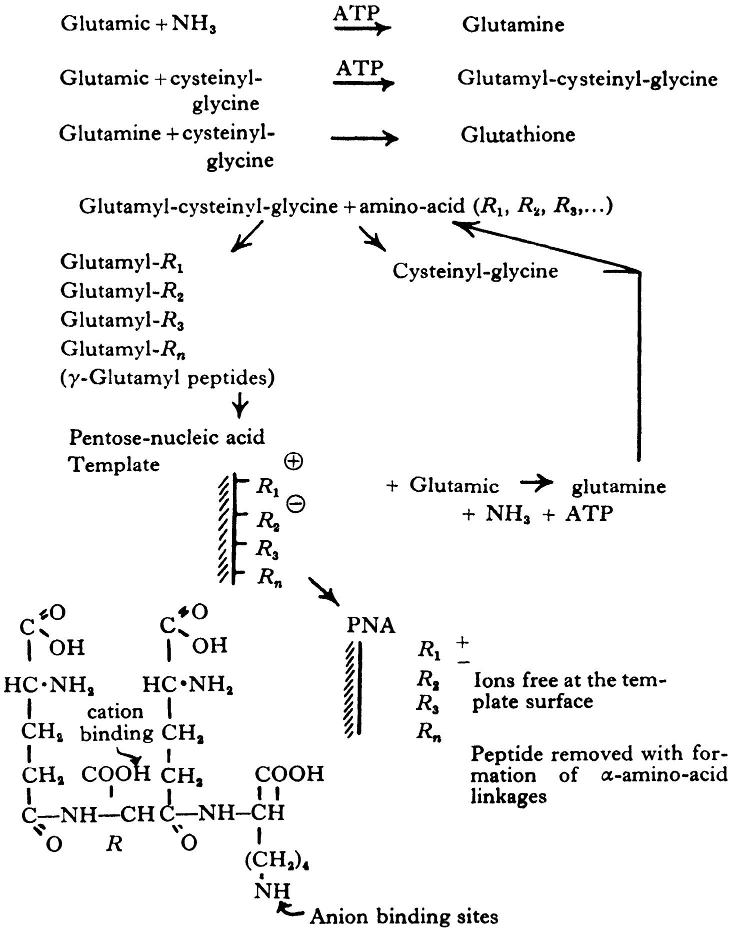
α-peptide links are to be formed and the sites hitherto available to bind anion and cation disappear. If one visualizes this event as occurring at, or near, the tonoplast surface it seems a not improbable outcome that the ions thus freed could be moved into the vacuole along a very short, but very steep, diffusion path or they could be actually released on the side toward the vacuole. Such a hypothesis admittedly involves much speculation. However, the hypothesis has the attraction of bringing together so much otherwise unrelated evidence that it is here presented in outline in the form of the following charts (Text-figs. 3, 4). Knowledge of the intracellular location of pentose nucleic acid is still limited. Caspersson (1950) stresses that pentose nucleic acid congregates at the nuclear membrane surface and here protein synthesis occurs but from the work of Commoner (1950) on plant cells it is not impossible that pentose nucleic acid may accumulate at cytoplasmic surfaces.

These ideas bear obvious resemblance to views held on the events that occur with contraction and expansion in muscle. In the resting muscle, with its expanded fibres K^+ and PO_4^{3-} are conceived to be bound in non-dialysable form, at negative and positive sites respectively. After the muscle contracts, energy being required to bring this about, K^+ and PO_4^{3-} are liberated. Thus at the protein surface ions are alternately bound and released concomitantly with expansion and contraction (Haurowitz, 1950, cf. p. 164).

Some other evidence lies behind this train of thought. Work on the effects of mineral deficiency in relation to nitrogen metabolism has emphasized the striking consequences of growing plants without sulphur. Under these circumstances protein synthesis is arrested, soluble nitrogen compounds accumulate and (in *Mentha*) among these soluble nitrogen compounds the two which attain very great concentrations in the cells are glutamine and arginine. This is consistent with a mechanism in which sulphur, as glutathione, is required to catalyse protein synthesis and, lacking sulphur, the mechanism becomes blocked at the point where some of the principal products that accumulate (glutamic acid$\rightleftharpoons$glutamine) would otherwise give rise to the variety of amino-acids required to give protein.

However, for ion intake the crucial evidence would be the recognition that ^{137}Cs could be bound in some organic carrier molecule that could be detected. Following a suggestion from the work of Bolton (1950) and Roberts *et al.* in a private communication, chromatography of anhydrous methanol extracts, obtained from lyophilized tissue cultures grown in media containing coconut milk and ^{137}Cs, showed that the caesium exists in at least two states which differ in their mobility in methanol on paper.

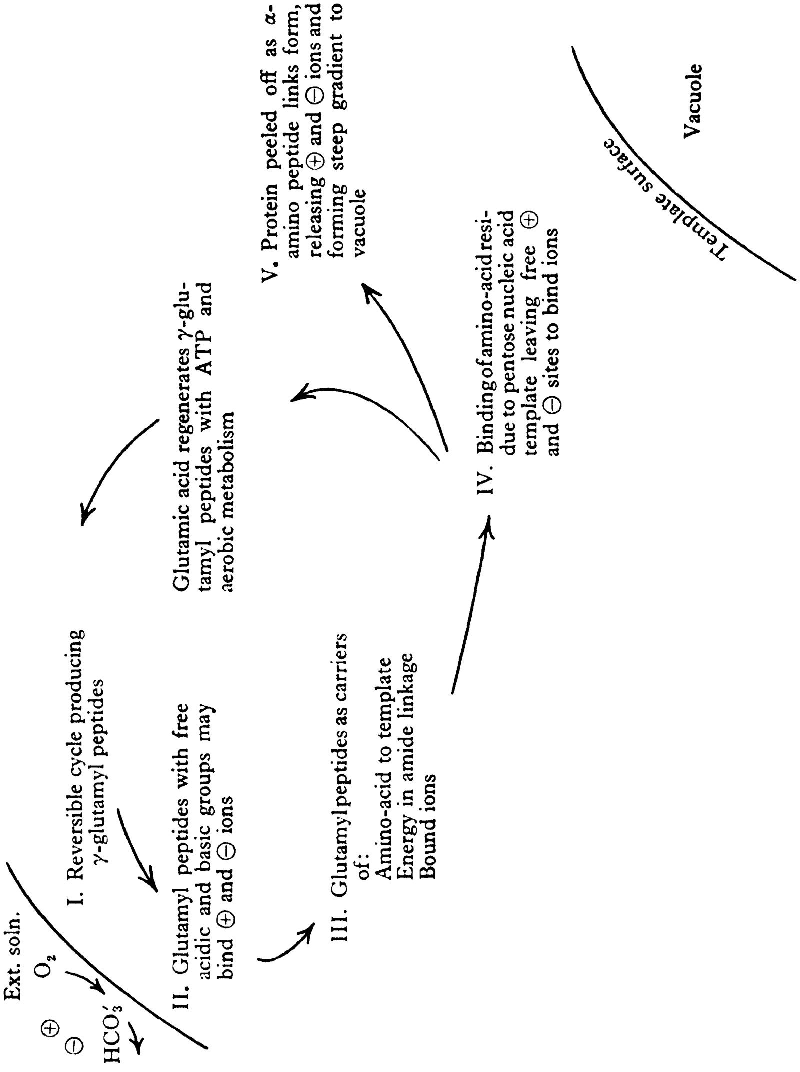
Spraying the methanol chromatogram with ninhydrin produced the pattern shown at Text-fig. 5. The more mobile ^{137}Cs component was closely superimposed by the pattern of ninhydrin activity in segment 2.



γ-Glutamyl-peptides may present to synthesizing surface:
- (i) Amino-acids
- (ii) Energy in amide linkage
- (iii) Associated ions:
    - Anions bound to basic amino-acids
    - Cations bound to more acidic amino-acids

Text-fig. 3.

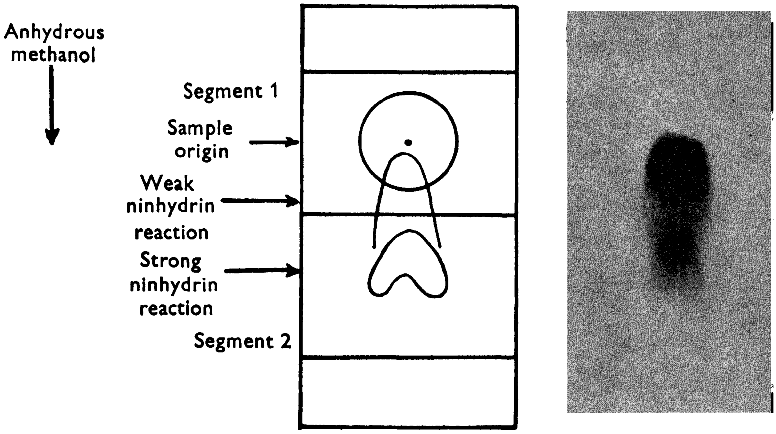
Similar segments 1 and 2 cut from the chromatogram were extracted with water and rechromatographed on a 5 × 5 in. paper using phenol: collidine-lutidine. Other segments from positions well above and below segments 1 and 2 were also treated in this way. These two directional chromatograms


Ext. soln.
⊕
⊖
O_2
HCO_3'
I. Reversible cycle producing γ-glutamyl peptides
II. Glutamyl peptides with free acidic and basic groups may bind ⊕ and ⊖ ions
Glutamic acid regenerates γ-glutamyl peptides with ATP and aerobic metabolism
III. Glutamyl peptides as carriers of:
Amino-acid to template
Energy in amide linkage
Bound ions
IV. Binding of amino-acid residue to pentose nucleic acid template leaving free ⊕ and ⊖ sites to bind ions
V. Protein peeled off as α-amino peptide links form, releasing ⊕ and ⊖ ions and forming steep gradient to vacuole
Template surface
Vacuole

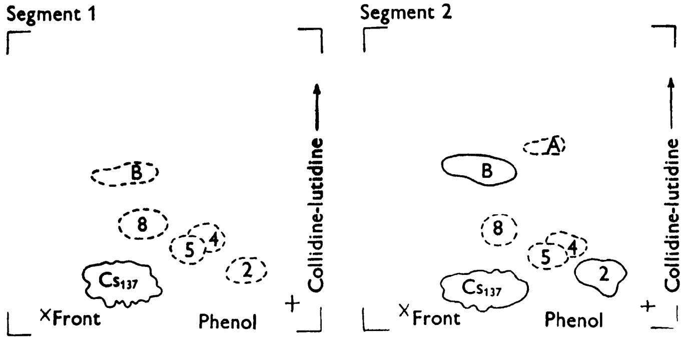
Text-fig. 4.

were then sprayed with ninhydrin and also treated with the starch-chlor-iodide method of Rydon & Smith (1952).

Although some traces of amino-acids were present in the original paper (washed only with methanol), the following results were quite clear.



Text-fig. 5. One-dimensional chromatogram in anhydrous methanol of a methanol extract of lyophilized carrot-tissue culture. 1, ninhydrin reactive compounds; 2, ^{137}Cs.



Text-fig. 6. Identification of ninhydrin reacting compounds that move in methanol alongside ^{137}Cs. Phenol: collidine-lutidine chromatogram of eluates from segments of methanol chromatogram.

When the two ^{137}Cs containing segments (1 and 2) of the methanol chromatograms are extracted and the extracts chromatographed in aqueous solvents both give one and the same ^{137}Cs spot (Text-fig. 6). Therefore there is the possibility that ^{137}Cs existed in the dry methanol extract as an organic complex, which broke down in the extraction and the more acid aqueous phenol. In this case one product of the decomposition could be aspartic acid from segment 2, for this substance appeared on the papers of Text-fig. 6 in amounts far beyond any possible error, and it now moved independently of the ^{137}Cs. Segments lower than 2 also showed that there were more mobile amino compounds in the methanol extract. The compound B detected by the starch-chlor-iodide test was not associated with the ^{137}Cs because it also appeared in the segment below no. 2.

As additional controls ^{137}Cs was chromatographed in methanol in presence of other salts (KCl, KNO_3, K_2SO_4) and a mixture of amino-acids. ^{137}Cs did not give two sharply defined spots (cf. Text-fig. 5) in any of these cases, and it ran more slowly than, and independently of, all the amino-acids and ninhydrin reacting materials.*

Therefore, the probability exists that ^{137}Cs occurred in the methanol extract of lyophilized carrot-tissue culture as a complex, decomposable by water, with a ninhydrin reactive material of which one product at least could be aspartic acid.

These observations are mentioned because they suggest lines along which further investigations of possible carriers of ions undergoing salt accumulation may be sought but not to claim that the present evidence alone is more than suggestive.

V. RESPIRATION AND SALT ACCUMULATION: THEORETICAL CONSIDERATIONS

If is not proposed to review all the recent papers that bear upon the relationship of respiration to salt accumulation. The following observations, however, are meant to relate the work here described to the interesting work of Robertson and of Lundegårdh to which it is so closely related.

The work of Robertson (1951 and references there cited) has been done with carrot-root tissue, mainly secondary xylem. After long washing and in nutrient-free solution the capacity of the cells for further growth and anabolism (protein synthesis) will be limited. The tissue as used by Robertson would seem to be in a somewhat comparable state to the carrot-phloem tissue which lacks the growth-promoting factors of coconut milk, i.e. it has a limited ability to grow, but this will be predominantly, if not exclusively, by enlargement of preformed cells. Early in the time drift the respiration is high and comparable with that of potato disks; later in the time drift it falls to a lower value. In this respect the behaviour of the carrot root resembles that of artichoke tuber (Steward & Berry, 1934). According to Robertson the respiration early in the time drift, i.e. at the initial high rate, is markedly cyanide-sensitive; later in the time drift it is less so.

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* Adding ^{137}CsCl to a methanol extract of lyophilized tissue gave the same chromatographic results as if the tissue had absorbed the CsCl. Therefore, the Cs-containing complex can be formed in an anhydrous extract of tissue.

Robertson has used the carrot tissue in this particular state, i.e. after it has adjusted to a steady but low level of metabolic activity, as the means to investigate the relation of respiration to salt intake. Certain features of this technique require to be noticed.

(1) The single salt solutions to which the tissue is exposed are relatively strong (up to 0·05 M)—these contrast with the very dilute solutions from which 'accumulation' often occurs.

(2) The determination of ion intake is almost wholly by the non-specific conductivity method.

(3) Where the papers, e.g. Robertson, Wilkins & Weeks (1951), Text-figs. 2, 3, refer to 'accumulation' of ions the data clearly relate to the amount of ion (or rather salt) absorbed from the external solution. Since the internal concentrations of the ions in question were not usually determined and are often considerable (as, for example, potassium, calcium, chloride) they would modify greatly the estimate of 'accumulation' (and therefore the work done), using the term 'accumulation' in its conventional sense.

With these points in mind the following features of Robertson's investigations on carrot tissue may be noted:

(1) Having adjusted to a low rate of respiration the tissue responds to the presence of a relatively high concentration (0·05 M) of salt by an increase in respiration which is steep at first. In the case of potassium salts the high level reached tends to be maintained; in the case of calcium salts it more quickly declines.

(2) After the initial increase the salt-induced respiration and the absorption of salt proceed concomitantly. (One cannot really tell from this alone that 'salt respiration' is causally related to the salt absorption, for it may be possible to devise situations in which the absorption of ions occurs without recourse to measurably increased respiration, as, for example, in the case of ^{137}Cs in very dilute solution.)

(3) The salt-induced respiration is cyanide- and carbon monoxide-sensitive, so these inhibitors retard both respiration and salt uptake by the tissue in this stage.

(4) The effects of dinitrophenols (loc. cit. pp. 258 et seq.) are, however, in this respect somewhat anomalous because they increase respiration without concomitant increase in salt uptake and may even, on the contrary, cause it to decrease.

(5) Using the tissue in the condition described, Robertson compared the oxygen uptake to the salt absorbed in the following way. For every molecule of oxygen absorbed, 4 atoms of hydrogen would ultimately be transferred, over pathways mediated by the appropriate dehydrogenase and cytochrome oxidase as terminal oxidase. Each electron transfer would

ultimately result in a hydrogen ion. The supply of hydrogen ions so produced furnishes the absorbing power for the cations to be absorbed. The basic scheme is that of Lundegårdh (1945), which visualizes the salt-induced respiration that is involved as proceeding over an iron-catalysed, cyanide-sensitive, respiratory system.

The data here reported on carrot and artichoke tissue—extending the body of data on potato tissue and roots already published—present on this view the following problems.

In dilute solutions (10^{-3} for KBr down to 10^{-9} for ^{137}Cs) the direct effects of the presence in the external solutions of the ions to be absorbed are either small or not measurable, as in the case of very dilute solutions of ^{137}Cs. In these cases, however, great accumulation (based on actual test of the tissue and the external solution) does occur, and it is determined by time and oxygen concentration in such a manner that it obviously proceeds *pari passu* with the aerobic respiration and metabolism of the tissue as a whole—not merely with a component of it due only to the *presence* of the salt.

In the case of both carrot and artichoke tissue there are clearly *two* distinct relationships to respiration that are to be considered. In nutrient solutions plus coconut milk, respiration is greatly stimulated and absorption of an indicator ion (^{137}Cs) proceeds in time concomitantly with growth and respiration, but the internal concentrations and the degree of accumulation attained, despite the increased aerobic respiration, are *reduced* in comparison with the non-dividing cells. *Per contra*, as the cells develop and pass out of the dividing state, or by withdrawal or deprivation of the coconut milk, though their respiratory intensity may decline, their attained *salt concentrations may increase*. This is another though dramatic example of the kind already noted (Steward *et al.* 1943) which suggests that the nature of the oxidative pathways by which the carbon dioxide emerges has a profound effect on whether it is, or is not, linked to the process of ion intake and accumulation. Previously stress has been laid on the fact that, to be effective in promoting salt accumulation, the carbon dioxide, and the oxidation by which it is produced, needs to be linked to protein synthesis. The data here recorded also stress that there is a profound difference between dividing, proliferating cells and cells whose growth is mainly, if not solely, by enlargement of their vacuoles.

It is, therefore, not possible to frame a hypothesis covering all the facts of ion accumulation based upon the direct intervention of oxygen uptake or carbon dioxide output *per se* in producing salt accumulation. The ultimate explanation requires a much more intimate understanding of the reactions that lead up to the final emergence of carbon dioxide and the transfer of hydrogen to molecular oxygen of the air, and of the way in which these reactions are used by the dividing and by the non-dividing cell.

Thinking in this general field has been influenced by the dramatic advances made since Lipmann (1941) outlined energy transfer through phosphorylation and, notably, by the knowledge that mechanical work in muscle can be visualized to flow from the energy actually donated to shortening muscle fibres as the muscle protein splits off phosphate from ATP.

In plant cells the *kind* of salt-absorbing system is clearly determined by the manner of growth of the cells in question (i.e. whether predominantly by division and self-duplication or by enlargement). For carrot and artichoke tissue, particularly, this is experimentally controllable by the use of the growth factors in coconut milk. However, within each metabolic pattern as thus determined there must be specific points, or reactions, at which the energy for the salt accumulation is furnished in a milieu in which the details of molecular architecture are conducive to it. But clearly there are in the overall mechanism two distinct steps, or types of process (stages I and II), and they each have a metabolic basis and a relation to a distinct and definite phase of growth in the cell.

Stage I (promoted by the coconut-milk factor) is typical of the cell in active division and in active multiplication of its self-duplicating parts. In this case the metabolism and growth are characterized by cyanide-insensitivity but great sensitivity to nitrophenols. Here the relation of ion intake to metabolism seems to be that the overall respiration promotes the synthesis of new ion-binding sites and in this sense respiration and salt accumulation are linked. If the nitrophenol acts by uncoupling phosphorylation (Loomis & Lipmann, 1948) without which growth and protein synthesis do not proceed, this is of more consequence to the mechanism of ion intake than its effect on respiration alone, for it is of no avail that carbon dioxide is produced, if the energy cannot be donated through phosphorylation to perform useful work.

When the cell ceases to divide and expands its vacuole, accumulation of ions in free solution (stage II) begins. During the process the activity of the growth factors that previously determined division has either expired, or is suppressed. The sensitivity of the system to cyanide is now greater, and at some point in the kind of metabolism that is associated with cell enlargement there must be a stage at which, through definite molecular arrangements in which energy is transferred, the secretion of ions into the vacuoles is negotiated. If Robertson's carrot tissue is to be regarded as predominantly in stage II it is suggestive that an *increased* respiration, caused by nitrophenols, did not, as expected under the Robertson-

Lundegårdh view, inevitably result in an increased ion intake. It is, however, possible to harmonize this otherwise perplexing result with many others in which increased respiration alone, that is, respiration which is not harnessed to growth and protein synthesis, fails to promote salt intake. The nitrophenol may act by dislocating the mechanism of energy coupling through phosphorylation, for it is only indirectly that respiration is in this way made effective in ion intake.

In the meristem and its derivative tissues all these events (stages I and II and their associated metabolic reactions) proceed in rapid and orderly sequence as part of the pattern of growth and differentiation. However, in evaluating the different materials used for experiment, whether these are excised roots, aerated potato discs, carrot explants with or without coconut milk, etc., it is important to understand their special relations to this overall pattern and to realize that no single explanation can possibly cover the behaviour of all.

It still seems, however, that the relationship that is most necessary to the understanding of ion accumulation is its relation to the processes of growth and protein synthesis. The speculations that have been advanced recognize that all of these essentially endogonic reactions are coupled ultimately with exogonic reactions of respiration. But, as shown by the work on tissue cultures, the details of metabolic coupling and the very nature of the ion-accumulation process that occurs are different in cells that are growing by division and in cells that are growing only by enlargement.

B. SALT ACCUMULATION IN THE PLANT BODY

I. CENTRES OF GROWTH AND ACCUMULATION

Since the primary processes of salt accumulation are characteristic of cells capable of growth and division, one may survey the plant body and define certain centres of growth and salt accumulation. From the evidence on tissue cultures one may now recognize that these centres of growth and salt accumulation will behave differently according as their chief characteristic is growth by cell division or by cell enlargement.

In the angiosperm plant body attention should, therefore, be focused upon the special problems that the following centres of growth and salt accumulation present.

The root. A gradation of salt accumulation along the axis of unbranched roots has been recognized and correlated with similar gradients of metabolic activity (Prevot & Steward, 1936; Steward, Prevot & Harrison, 1942; Machlis, 1944). While it is true that these gradients dealt with segments of root, in which those nearest the tip contained a larger number of smaller cells,* it is still true that *per unit of water* the concentrations of absorbed ion followed a well-defined pattern along the root axis which is also recognizably correlated with the gradient along the axis of respiratory intensity *per unit water content*. The behaviour of the root may now be re-examined by reference to some experiments made with radioactive isotopes. These experiments again raise the question of the gradient of salt accumulation along the axis, the contrasted behaviour of the root apex, or meristem, and of the regions of cell elongation and differentiation, and also the problem of the mechanism of secretion into the stele.

The shoot apex. As the shoot apex produces leaf elements in orderly sequence they each pass successively through their developmental sequence. This sequence includes a brief phase in which cell divisions predominate followed by the 'Sach's grand period of growth', in which vacuolation and extension predominate as growth in size rises to a maximum rate and subside as the organ reaches maturity.

Entry of salt into the leaf, however, demands, first, *access* to salt via the vascular system and then the ability to *accumulate* it, whether this is done by virtue of the properties of growing-dividing or of growing-extending cells. Also reference should be made to the much neglected fact that while the leaves present on the axis at any one time on a herbaceous angiosperm shoot represent a developmental series, they are also to be regarded as more closely connected within vertical ranks or units, i.e. orthostichies. Within each orthostichy the possibility exists that the stimuli and interrelations which determine salt accumulation can operate in a more intimate fashion among leaves which are more directly connected by vertical vascular strands than throughout the plant body as a whole.

Also the problem of access to salt, the means whereby the solutes are drawn off laterally from the axis into the lateral organs, assumes a different aspect according as one considers it in terms of the organization of the herbaceous dicotyledon or of the monocotyledonous plant body.

Perennial woody dicotyledons also present their special problems. Here attention may be focused upon the role of the cambium as a centre of growth and salt accumulation in the axis and as an active agent in longitudinal and lateral movement of salt. The role of resting and active buds and the effect of the periodicity in their development requires to be evaluated in terms of their ability to absorb and accumulate salt.

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* For a dicotyledon root Robinson & Brown (1952) state that the number of cells per segment increases up to 2 mm. from the tip, the volume per cell up to about 12 mm. from the tip.

The following summary will make brief reference to investigations in this field made upon the following plants and organs:

(1) the uptake of ^{137}Cs by detached and attached roots of *Narcissus* with special reference to the longitudinal gradation of accumulation along *single* roots (making reference to experiments with R. Overstreet and S. M. Caplin, and F. K. Millar);

(2) the intake of bromide by the shoot of *Cucurbita* (with A. G. Steward);

(3) the intake of ^{137}Cs by the shoot of *Narcissus* (with S. M. Caplin);

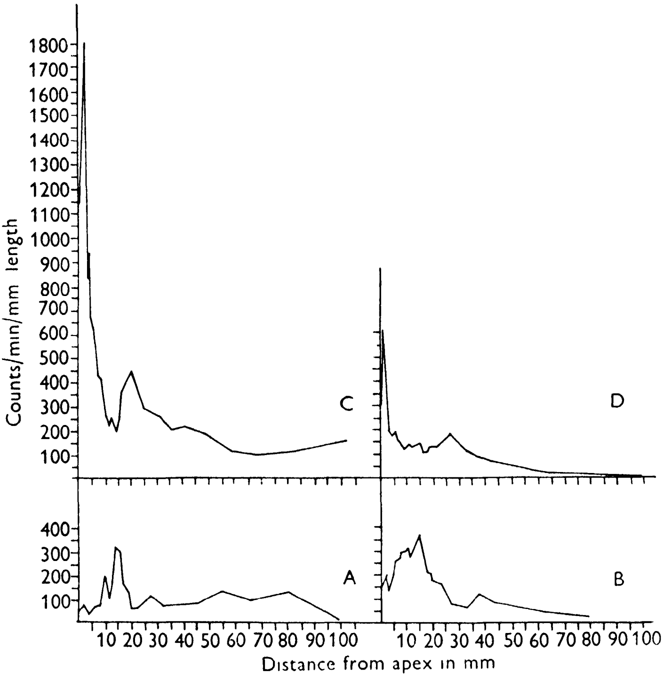
(4) the intake of bromide by the shoot of *Populus* with special reference to the role of the cambium and intake by the growing buds (with J. A. Harrison);

(5) direct absorption at the cambial surface of *Tilia* and of *Acer* (with B. M. Pollock);

(6) the intake of ^{137}Cs by the buds of *Acer* (with B. M. Pollock and F. K. Millar).

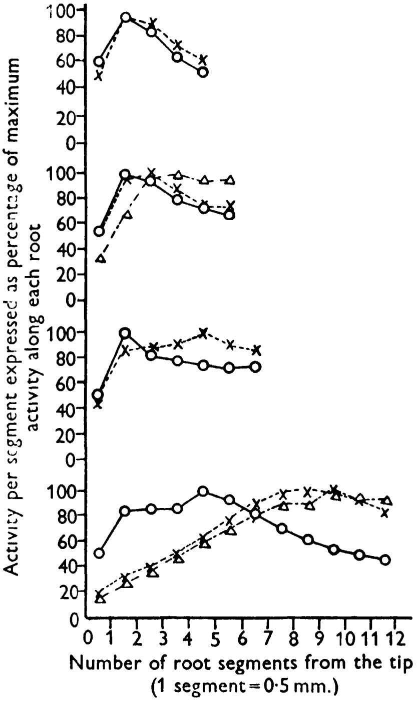
Absorption of ^{137}Cs *by* Narcissus *roots.* Overstreet & Jacobson (1946) used carrier-free radioactive isotopes and determined the absorption of these along the axis of single roots. Working at low temperatures (order of 2° C.), where metabolism would be at a minimum, they attributed the intake that they encountered to a non-metabolic absorption, or binding, which they regarded as the prelude to the metabolically determined accumulation to follow. Thus, even in non-dividing cells, Overstreet identified what would seem to be a transient phase in ion intake which is comparable (in the sense that it depends on ion binding) to that which persists when cells remain in the permanently dividing state, as in the tissue cultures already referred to. However, work with *Narcissus* roots, selected because they do not branch and because they grow well in water, produced some unexpected results. Experiments made in collaboration with Drs Overstreet and Caplin (see Steward, 1948) revealed that the gradients of ^{137}Cs absorption along the axis of *single* roots of *Narcissus* were very variable. In some roots the highest concentrations were obtained near, but just behind, the tip; in others the maximum absorption occurred many millimetres, even up to 2 cm., from the tip. Apparently a large random sample of roots will reproduce the smooth longitudinal gradation of accumulation that Prevot & Steward (1936) described, but this is only statistically true, and *individual roots* may deviate widely from this 'ideal' behaviour. The range of differences encountered with isolated roots taken from the same bulb at the same time may be seen in Text-fig. 7.

It is evident that even when the maximum absorption occurs near the apex, it still occurs a few millimetres behind the root tip. Also, superimposed upon the otherwise smooth basipetal gradation of accumulation, there are secondary maxima which may occur at almost any point, even too far back from the tip for their association with growing or dividing cells to be readily plausible. The radio-autographs of longitudinal half-sections of *Narcissus* roots give direct, visible evidence of this phenomenon (Pl. 1, fig. 1). This effect was so surprising when it was first encountered that it suggested the possibility that the concentrations of absorbed ion in the cells of the root are not static but that 'waves' of absorption may pass along a root. In this way the point of maximum concentration may migrate along the axis of the root, though it naturally occurs most frequently and exists for the longest time interval near the tip.



Text-fig. 7. Relationship between absorption of ^{137}Cs and distance of root segment from apex. All root attached. Absorption period 17 hr. Temp. 19° C. in dark.

Experiments have been made by Miss F. K. Millar (1953) in the attempt to settle this point by a technique which allowed her to trace out the distribution of ^{137}Cs along an attached root and then to follow the changes that occur with time. The full data and technique cannot be given here, but it must suffice to say that evidence was obtained that the point of maximum accumulation, first located at or near the root apex, could migrate along the axis of an *attached* root, and its rate of backward movement was of the order of 2–3 mm./48 hr. (Text-fig. 8). In these experiments, for reasons dictated by the technique, the leaves were removed from the bulb and the roots were in nutrient-free solutions of carrier caesium. Therefore these changes, i.e. the redistribution of the accumulation of previously absorbed ^{137}Cs with time, refer to the maturation of the preformed tissues of the root. Growth in length by the formation of new cells did not occur appreciably during these experiments.



Text-fig. 8. Linear distribution of absorbed ^{137}Cs in roots of *Narcissus*; the effect of time after absorption. A. Root excised before absorption. —O— distribution of ^{137}Cs at end of absorption period; - - - × - - - distribution of ^{137}Cs 20·5 hr. after absorption period. B. Root attached. —O— distribution of ^{137}Cs 6·5 hr. after absorption period; - - - × - - - distribution of ^{137}Cs 27 hr. after absorption period; — —△— — distribution of ^{137}Cs 54·5 hr. after absorption period. C. Root attached. —O— distribution of ^{137}Cs 3 hr. after absorption period; - - - × - - - distribution of ^{137}Cs 51 hr. after absorption period. D. Root attached. —O— distribution of ^{137}Cs at end of absorption period; - - - × - - - distribution of ^{137}Cs 48 hr. after absorption period; — —△— — distribution of ^{137}Cs 69 hr. after absorption period.

It is clear, therefore, that the root responds to stimuli which regulate its salt accumulation but which are as yet only incompletely known. The cells of the root meristem should resemble the 'growing-dividing' cells of the carrot-tissue cultures and thus owe their salt intake to stage I of the accumulation process, in which stoichiometrical ion-binding may predominate, but in which the highest *concentrations* are not necessarily produced. The drop in ^{137}Cs content in the apical segment is consistent with this (Text-fig. 8). The 'growing-extending' cells, in which divisions are less frequent, would be expected to attain higher concentrations accumulating their salts by stage II of the absorption process and secreting them into their expanding, aqueous vacuoles. Cells at the height of their growth by extension, just behind the meristem, should thus attain the highest concentrations, and indeed they often do.* But even so, there are maxima of absorption superimposed upon this 'ideal' behaviour and which exist so far from the tip as to suggest that other effects intervene to determine the absorption that may occur in these regions of the root. These 'other effects' are as yet unexplained except that they are probably part of a periodic or 'wave-like' movement that locates the point of maximum accumulation in the maturing cells of the root at different distances from the root apex at different times.

The more strongly absorbing attached shoot in the light has an unparalleled ability to deplete an attached root of its absorbed ions (Hoagland & Broyer, 1936). This depletion of the root by the shoot seems to occur more readily from near the tip (Steward *et al.* 1942). The problem of ion secretion into the stele and of their removal to the shoot seems still to be a complete mystery, except that it involves metabolism and respiratory energy; for in this respect the root is subject to regulatory control by the shoot, and the basis of this is still unknown.

The relative accumulation in leaf, stem and root. Text-figs. 9 and 10 show the concentration and total amounts of ions absorbed in the different regions of the plants named. The technique here is to plot the concentration (quantity per unit fresh weight or unit weight of water) as ordinate and the fresh weight of the sampled region as the abscissa, so that the height of each histogram is a measure of concentration and the area of the resultant rectangle is a measure of the total amount in the sampled region.

In *Cucurbita* and for bromide the relative order of ion accumulation is root < stem < leaf, and in the stem the internodes higher on the axis tended to attain the higher concentrations (Text-fig. 9). In *Narcissus* the order obviously is root > crown > leaves (Text-fig. 10).

We are clearly not able, as yet, to specify what determines the ability of one organ to deplete another, nor can we venture to explain the differences

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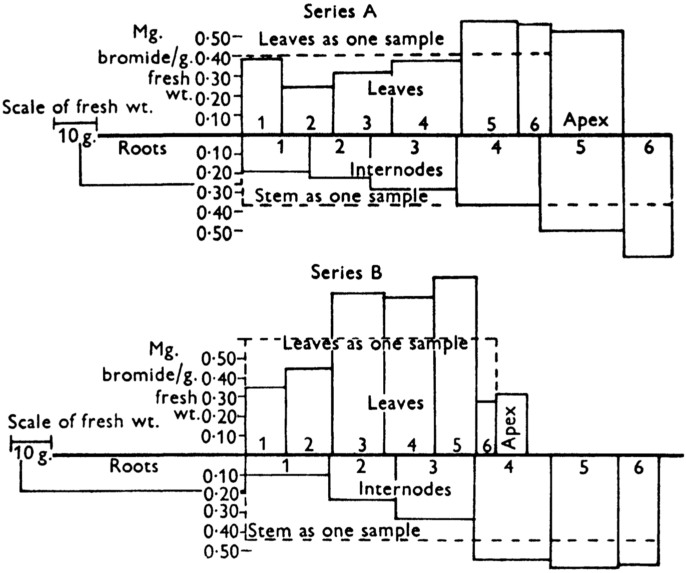
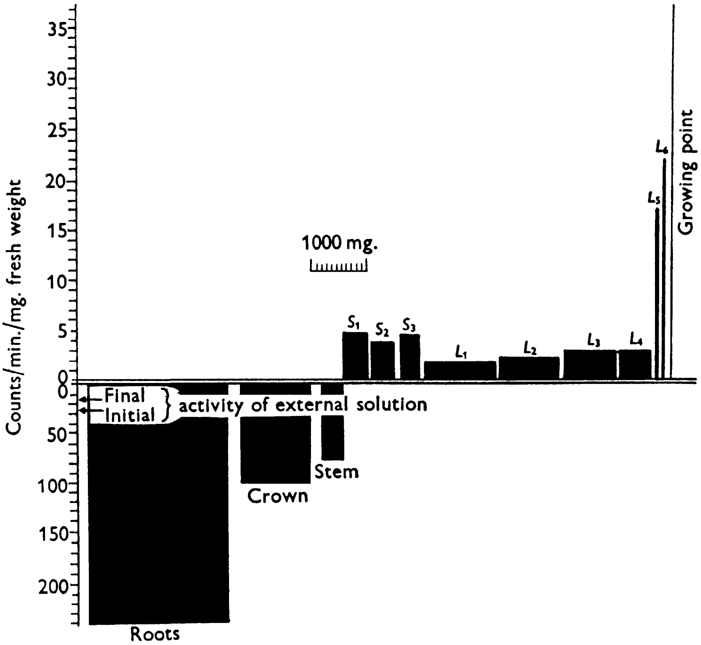
* Brown has rendered a service by showing that various properties reach their maximum value *per cell* coincidentally, or nearly so, with the attainment of maximum cell size, notably protein-N content and the activity of certain enzymes (Robinson & Brown, 1952). There can be little question also that the interval in which the cell embarks upon and reaches its maximum intake of salt spans its own growth. It is still true, however, that the 'intensity' of the salt absorption is best measured by referring it, as a concentration, to the quantity of water in which it occurs.



Text-fig. 9. Distribution of bromide in *Cucurbita pepo*. Plants of series A received KBr via their roots during periods in which the shoots were in the light; plants of series B received KBr via their roots but only during the alternating periods in which their shoots were in the dark. Light and dark periods were adjusted to 12 hr. each.



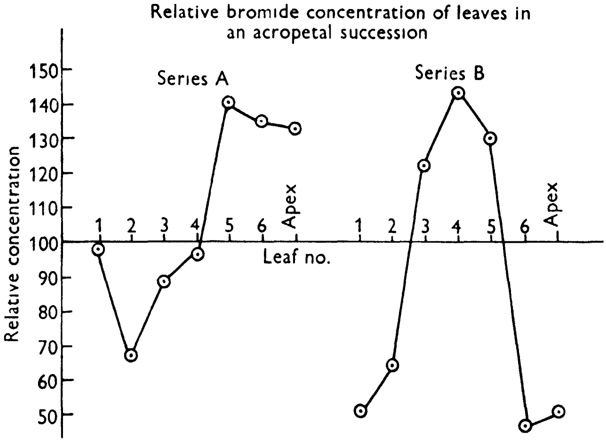
Text-fig. 10. Absorption and distribution of ^{137}Cs in *Narcissus*. *SS*, sheath leaves in acropetal succession; *L*, expanded leaf with sheathing base; stem, axis of lateral bud bearing *S* 1–3 and *L* 1–6; crown, flattened main axis of bulb.

between the behaviour of *Cucurbita* toward bromide and *Narcissus* toward ^{137}Cs in this respect.

However, it is possible to analyse the accumulation in the leaf in relation to its development.

Absorption and accumulation in the leaves of herbaceous plants. Cucurbita, which grows sympodially, is the selected example of a dicotyledon and *Narcissus* the selected example of a monocotyledon.

The *Cucurbita* experiments were performed in such a way that the shoots of one series (A) received potassium bromide via the roots at the time that their shoots were in the light; the other series (B) received potassium bromide via the roots while their shoots were in the dark.*



Text-fig. 11. Relative bromide concentration of leaves in an acropetal succession. Entry of bromide into the leaf of *Cucurbita pepo*. (Relative concentration in each leaf equals the concentration in the leaf divided by the average concentration in the leafy shoot as a whole.)

Text-fig. 11, series B, shows that in leaves of the first phyllotactic series of a *Cucurbita* seedling the intake of bromide by the leaves reflects their own growth and development since the *leaves which are expanding most have the highest concentration of bromide*; the curve of bromide absorption against leaf number on the axis clearly reflects the stage of each leaf relative to 'Sach's grand period of growth' for that leaf.

However, superimposed upon this pattern are the following effects. When, as in series A, the leaves are in the light and *simultaneously the roots have access to salts*, the apex grows, absorbs salt (potassium bromide) direct from the roots and also depletes the lower leaves of previously absorbed

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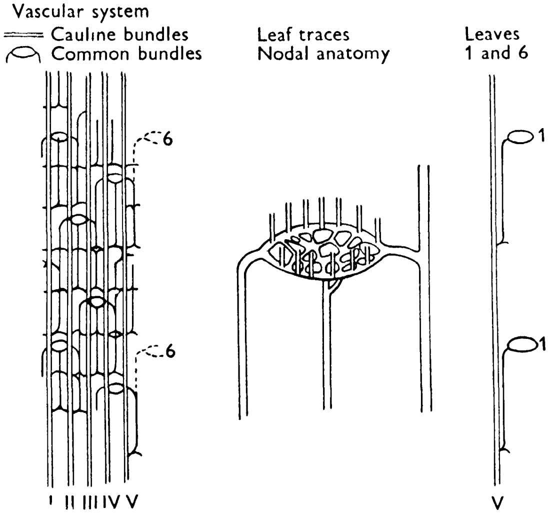
* The periods of light and dark were the same duration (12 hr.) and the plants were transferred as necessary from culture solution with bromide to culture solution without. The experiment involved several such cycles.

salts so that they become able to take in more of the indicator ion (bromide). These facts become intelligible when it is realized that the leaves 1, 6, 11, etc., of *Cucurbita* constitute a vertical series, or orthostichy, and these leaves are in direct connexion via one of the five cauline bundles (Text-fig. 12).

Thus the leaves in one orthostichy constitute a closely integrated nutritional system. As each new leaf is cut off from the apex and is added to the series it passes through its own sequence of development and of primary salt accumulation. This salt intake proceeds slowly at first, when cell division predominates, gathering momentum and reaching high concentrations at the height of its own expansion. Thereafter, further intake of salt declines as expansion subsides unless, in response to the competition from growing leaves above, the leaf in question is depleted of total salts and thus acquires a vicarious ability to absorb again from the root, when the supplies of absorbed salt are restored.



Text-fig. 12. The vascular pattern of *Cucurbita pepo*, showing direct connexion of leaves 1 and 6 via cauline bundles. Each leaf receives three leaf traces which fuse into a network at the nodal plate. Of these three, one springs directly from the cauline bundle.

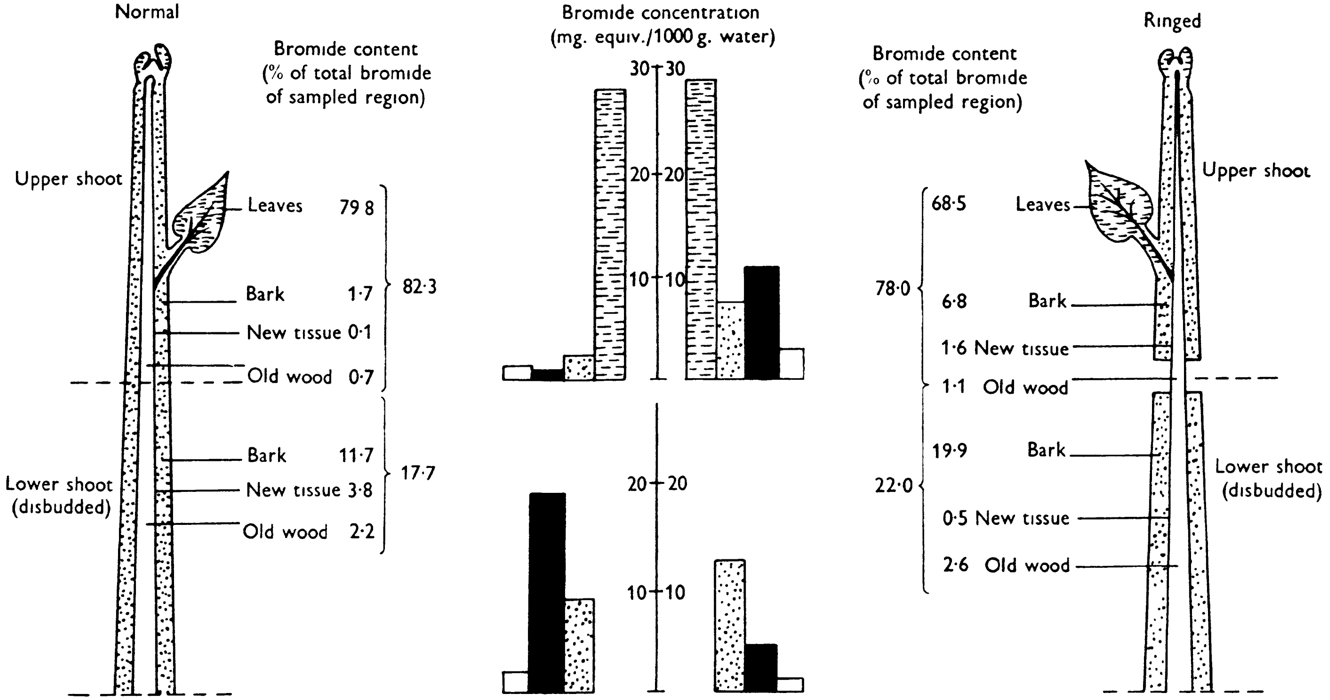
Before the bromide could be accumulated in the leaves of *Cucurbita*, it had to be drawn off laterally and enter the vascular system of the leaf. This only occurred extensively in the light under the conditions in which the leaf itself grew. The mechanism which enables salts to enter the vascular supply to the leaf is best postponed until the conditions in a tree are described. However, in dicotyledons the vascular cambium, by its very potentiality for growth, commands attention as a region of potential salt accumulation in the stem and as the means by which salts are directed by the ebb and flow of its activity to the lateral buds.

Attention in the monocotyledon should be focused upon the intercalary growing region at the base of each leaf; for this is not only the means by which these linear leaves grow, but it can also be a centre of salt accumulation (at the expense of the solutes in the stele) from which salt is accumulated and diverted to the leaf. Experiments in which ^{137}Cs was allowed to enter the shoot of *Narcissus* substantiate this view, for ^{137}Cs accumulated strongly in the basal meristematic region of both the leaves and sheathing scales of the bud (Pl. 1, fig. 2*a*, *b*). In these figures the sheathing leaf bases are shown 'opened out' and as mounted for the radio-autograph.

In the shoot of *Narcissus* the *concentration* of absorbed ^{137}Cs in leaves increased markedly the nearer they approach to the apex of the shoot (Text-fig. 10). However, even the smallest segment tested does not permit one to distinguish between the meristem proper and the rapidly elongating, differentiating cells to which it gives rise.

Centres of salt accumulation in a tree. In the shoot of deciduous woody dicotyledonous trees, each lateral bud, as a potential growing region, is a potential region of salt accumulation which, granted its ability to gain access to salts, will recapitulate the potentialities of the terminal growing point. Buds, however, arise in isolation in the axils of leaves, and their vascular supply does not immediately integrate with that of the main axis. In trees it is a common condition that the bud rapidly becomes dormant in the year of its formation; in its dormant condition absorption of salts is at a minimum. In the ensuing spring, with the onset of the familiar but mysterious process of 'bud-break', a renewed activity in the vascular cambium emanates from the bud and spreads basipetally downward. In the outcome, the effective vascular supply to the bud integrates with the vascular tissues in the stem, which develop, not in the year of the formation of the bud, but in the year of its growth. The significance of these facts in the internal nutrition of trees has been largely overlooked; but their pertinence was revealed by an investigation into the uptake of bromide by a population of poplar trees (*Populus nigra*) throughout one annual cycle of growth in England (Harrison, 1938).

By appropriate experimental devices (not here reported) it was possible to show that in the dormant condition there was a slow upward, probably diffusive, spread of solute in the old wood. In the spring, however, the active cambium becomes a growing region, active in the accumulation of solutes, which are drawn laterally from the old wood within (Text-fig. 13). Not until the growth and differentiation of vascular elements develops



Text-fig. 13. Centres of salt absorption in the shoot of *Populus*. The normal shoot is disbudded below the level shown, but the cambium is stimulated by the growing leaves above. As the buds grow, most of the bromide in the shoot goes to the leaves; the cambium region is depleted of the bromide, which it absorbs from the wood within. In the lower region of the stem—without leaves—the cambium region accumulates markedly more bromide than the old wood within. The ringed shoot is disbudded below the ring. Leaves grow less in ringed shoots. Some residual accumulation of bromide in the cambial region remains even above the ring. Below the ring, the cambium is much less active in the disbudded shoot, so that, even though there are no leaves to absorb it, there is relatively little bromide accumulated in the cambial region.

backward from the bud and passes downward in the stem, in the current year of growth, does any appreciable entry of salt into the bud occur; but thereafter it continues smoothly throughout the period when the bud grows and develops. The data show that the leaves of *Populus* could reach an appreciable size (4·4 g.) before intake of bromide from the old wood became effective, but thereafter intake and growth in size went hand in hand. The regression equation of growth (x = g. fresh weight) on total bromide content (y = mg.equiv.) is given by $y = 0{\cdot}023x - 0{\cdot}101$.

Thus, the vascular cambium in a tree is to be recognized as a prominent region of growth and of salt accumulation. A primary function of the cambial region is to absorb salts from the dilute solution in the old wood within and transfer them in the developing vascular traces to growing buds immediately above.

The knowledge that the cambium of woody dicotyledons acts as an absorbing and accumulating region suggested the possibility that a radioactive isotope could be applied directly to the exposed external surface of the cambium at the time of its activity in the spring. When this is done, by building small cups around the cambium exposed by removing a window of bark, the absorbed isotopes move preferentially to the bud immediately above and on the same side of the shoot as its point of application (Steward, 1948).

Following upon this technique, experiments have also been made to indicate the kind of absorption mechanism by which the young expanding leaves of maple absorb ^{137}Cs (Pollock, 1950; Millar, 1953). The following points are particularly relevant to this discussion.

(1) Entry into the very young leaf of ^{137}Cs supplied via the cambium surface is almost completely confined to leaves in the light. This was true even though the leaf was obviously expanding and so might not have been expected to absorb by the method typical of actively dividing cells.

(2) Entry of ^{137}Cs into the young leaf is affected by the presence of added carrier in ways which are consistent with the view that here also, as in the rapidly dividing tissue cultures, the ^{137}Cs is being stoichiometrically bound to cation-binding sites, probably on nucleic acid surfaces. Also, such attempts as have been made to apply *both* ^{137}Cs *and* respiratory inhibitors to the cambium surface suggest that, in its response to inhibitors (comparative insensitivity to cyanide), the tissue of the young leaves of the buds of maple resembles the behaviour of the growing-dividing cells rather than of the growing-extending but non-dividing cells.

These observations would, however, become intelligible if new binding sites (as at a nucleic acid template surface for protein synthesis) were being multiplied in these leaves *in the light*.

The role of the root apex, the shoot apex, the developing leaf buds, the vascular cambium and the intercalary meristems of monocotyledons as centres of growth and salt accumulation in the plant body is, therefore, demonstrable. All the special problems that each presents can obviously not be dealt with here, or even on present knowledge. The information that has accrued from the investigation of cells at different stages of their development tells something of the diverse ways in which cells appear to use their metabolism to absorb and accumulate their salts. Each region, however, can hardly operate in isolation, for shoots deplete roots, leaves in the same orthostichy interact with each other and the active cambium accumulates ions from the dilute xylem fluid within and supplies them, via the current year's growth, to the buds above. Leaves in one orthostichy constitute a more closely knit nutritional unit than the leaves of the whole shoot. The fact that this complex pattern is controlled and integrated is evident; the method by which it is accomplished is, however, totally unknown. Though we now have some idea how a given cell absorbs its solutes from dilute solution in the first place, we have no idea of the nature of the stimulus that prompts that cell to part with those solutes so that they may be directed to even more strongly accumulating cells elsewhere in the plant body.

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EXPLANATION OF PLATE

Fig. 1. Radio-autograph of longitudinal half-sections of *Narcissus* roots containing ^{137}Cs. Exposure 10 min. on X-ray film. Six roots of *Narcissus* from a bulb grown in tap water.

Fig. 2. (*a*) Radio-autograph of dissected *Narcissus* shoot. Exposure 30 days on X-ray film. Younger leaves accumulated relatively more caesium than older ones. The sheath portion of a leaf accumulated almost all the caesium taken up by the leaf. The sheath portion opposite the blade accumulated relatively more than the sheath portion below the blade. (*b*) *Narcissus* shoot dissected and mounted for radio-autograph. Lower row: sheath leaves, the outermost one at right. Upper row: expanded leaves; oldest expanded leaf on right, youngest leaf enclosing the stem growing-point on extreme left.

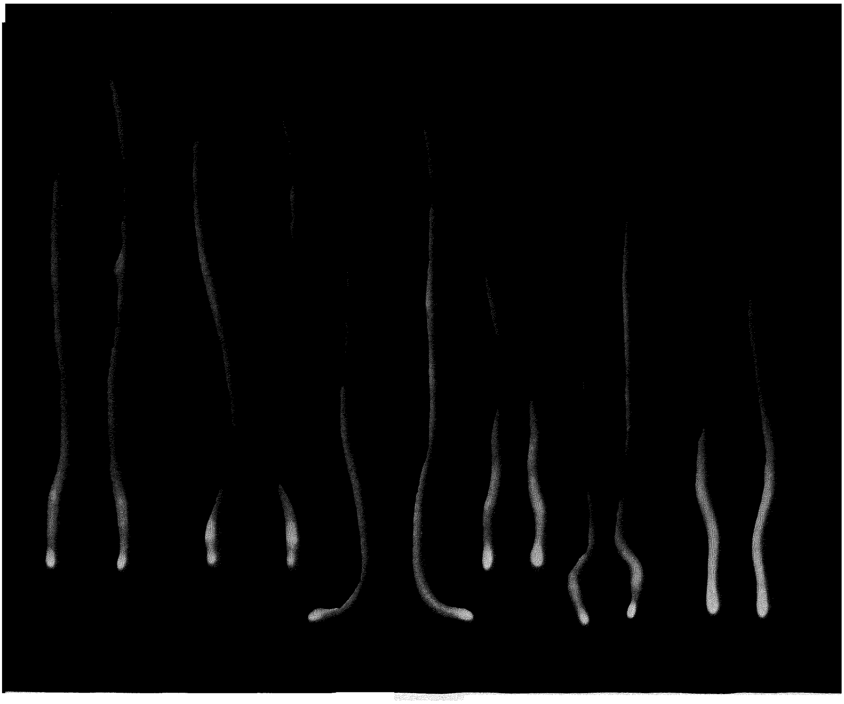

Fig. 1.

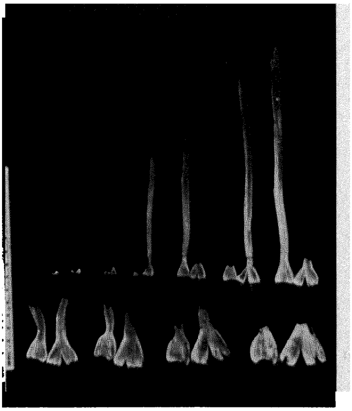

Fig. 2(*a*).

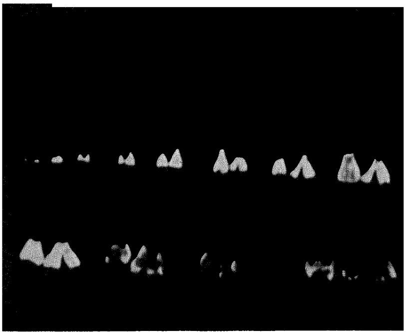

Fig. 2(*b*).

For explanation see p. 406

ACTIVE TRANSPORT OF INORGANIC IONS

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The last two decades have seen a greatly increased interest in the phenomenon of active transport of inorganic ions. It has been accepted by most workers in the field that the peculiar ionic distribution between living cells and their surroundings as well as the secretion by glandular tissues must involve the specific transport one way or the other of certain ion species. Transport which is energized by the living cells is termed active transport. This definition is not entirely unambiguous, however, and it might be appropriate at the outset to discuss briefly how the term 'active transport' should properly be applied. We have to admit that, despite the considerable effort put into the study of the behaviour of the inorganic ions, there is still very much lacking in our understanding of the mechanisms by which they are handled in the organism. Apparently the best thing we can do now is to systematize our ignorance, or, in other words, to sort out the features of the behaviour of the ions which can be explained by known physico-chemical forces; what remains then is pooled under the heading 'active transport'. Consequently, as our understanding of the processes has improved, there has been a tendency to restrict the use of the term 'active transport'. Let us, for example, consider the distribution of cations between the muscle fibre and its surroundings. The concentration of potassium in the fibre is perhaps 40 times higher than in the medium. At first sight this requires some active transport mechanism pushing potassium into the fibre. As pointed out originally by Dean (1941) the high potassium concentration, however, is probably the result of the functioning of a transport mechanism, a 'sodium pump', which pushes sodium ions out of the fibre, thereby creating the potential difference across the fibre membrane which in turn forces the potassium ions to enter in excess over the concentration outside. Obviously, both the high potassium concentration and the low sodium concentration in the fibre are the results of the functioning of the sodium pump, but only the movement of sodium should be termed 'active transport'. The sodium ions move from a low concentration and a negative potential to a high concentration and a positive potential, a process which requires work on the part of the cell. The potassium ion, on the other hand, seems to be practically in Donnan equilibrium across the fibre membrane (Boyle & Conway, 1941). Thus we speak of active transport when the ion is transferred against an electrochemical potential gradient, and one might indeed limit the use of the term to such cases (cf. Rosenberg, 1948; Ussing, 1949*b*). There is, however, no reason to believe that the mechanism which is able to transport a certain ion species against an electrochemical potential gradient should be unable to assist in transporting the same ion species 'down hill' under a different set of experimental conditions. Logically the latter phenomenon should also be considered active transport. The only question is then whether it is experimentally possible to demonstrate that an active transport process is 'assisting' the spontaneous diffusion of an ion.

Let us consider first the behaviour of a non-electrolyte, for instance, glucose. This substance is supposed to diffuse through a living membrane which separates an 'outside solution' from an 'inside solution'. The glucose concentration in the outside solution is maintained constant at c_o, whereas that of the inside solution is maintained constant at c_i ($c_o > c_i$). When the steady state has developed we have by Fick's law that the total amount of glucose passing from the inside to the outside solution per unit time is $M_{\text{out}} = k.c_i$. Similarly, the influx is $M_{\text{in}} = k.c_o$. We thus have

$$M_{\text{in}}/M_{\text{out}} = c_o/c_i. \tag{1}$$

In other words, if only diffusion is operative, the flux ratio has to be equal to the concentration ratio for the diffusing substance. In principle the flux ratio can easily be measured using two isotopically labelled types of glucose to measure influx and efflux, respectively. One may, for instance, add glucose labelled with heavy carbon, ^{13}C, to the inside medium and calculate the efflux from its rate of passage into the outside medium. Simultaneously, the influx could be measured by aid of ^{14}C-labelled glucose added to the outside medium.

It is easily seen that the flux ratio ($M_{\text{in}}/M_{\text{out}}$) cannot be larger than the concentration ratio (c_o/c_i) if the process is one of simple diffusion. If the flux ratio is nevertheless found to be larger than the concentration ratio, we would speak of active transport.

For electrically charged particles Fick's law does not apply. In spite of this it can be shown that an equation formally identical with (1) is valid for ions that diffuse passively through a membrane. The equation may be written

$$M_{\text{in}}/M_{\text{out}} = \frac{f_o}{f_i}\frac{c_o}{c_i}e^{zFE/RT}, \tag{2}$$

where f_o and f_i are the mean activity coefficients, c_o and c_i the concentrations of the ion in question in the outside and inside solutions, respectively, and E is the potential difference between the two solutions (cf. Ussing, 1949*a*, Teorell, 1949).

As soon as the steady state has been established, this equation applies for any ion species passing by simple diffusion only. The equation holds not only for a homogeneous membrane, but for any number of superimposed layers, or for a membrane where the properties vary continuously all the way through.

If the parameters occurring in equation (2) are available for measurement, we are in a position to decide whether or not the passage of the ion through the membrane is due to active transport.

Until now we have been considering a membrane which constitutes a non-aqueous phase. If we are dealing with a pore membrane, the situation is complicated by the fact that there may be a net flow of solvent through the pores. If this is the case, the flowing solvent will exert a 'drag' upon all diffusing particles. Such a drag would speed up the ion flux in the direction of solvent flow while opposing the flux in the opposite direction. A discrepancy brought about by such a drag between the flux ratio found and that calculated according to equation (2) should not be considered evidence of active transport. This is obvious if the flow of solvent is due to a hydrostatic or osmotic pressure difference across the membrane. But even if the solvent flow is due to processes in the membrane itself, it would be preferable to speak of active water transport rather than active ion transport, as long as the deviations from equation (2) were due to the drag only. The drag force is non-specific, just as the electric potential force is. The problem, whether or not the living membranes, and the cell membranes in particular, have pores, thus attains considerable interest. As is well known Collander (1937) considered his experiments with the diffusion of non-electrolytes into Characean cells as indicating a lipoid-pore structure of the cell membrane. This interpretation has been criticized by Davson & Danielli (1943), who proved theoretically that similar results might be obtained with a continuous membrane in which the molecules dissolved. On the other hand, it was pointed out by the present author (1952) that in case pores are present in a cell membrane, its water permeability, as measured by the rate of osmosis, would come out higher than its permeability as measured by the rate of diffusion of isotopic water. This is a simple consequence of the fact that flow through pores is dependent upon the pore size (compare Poiseulle's law) as well as upon the total number of pores. Diffusion, on the other hand, depends solely on the total area available to diffusion. Experiments in our laboratory by Zeuthen & Prescott (1953) did indeed show that, according to the criterion just mentioned, several types of egg-cell membranes must have pores. Nothing can be said as yet about whether pores are also present in the ordinary cells of the animal body, but experiments comparing the osmotic and diffusion permeabilities of the amphibian skin to water do indicate the presence of pores in this structure (Koefoed-Johnsen & Ussing, 1953), and the experiments performed by Visscher, Fetcher, Carr, Gregor, Bushey & Barker (1944) on the rate of passage of heavy water through the intestinal wall may be interpreted as indicating the presence of pores in the intestinal mucosa.

In organs where there is a considerable net flow of solvent across a membrane we should be prepared to meet with deviations from equation (2) brought about by the solvent drag. The solvent drag is proportional to the linear rate of flow of solvent through the pores through which the ion in question diffuses, and inversely proportional to the diffusion coefficient of the ion in water. It is relatively easy to evaluate the drag effect as long as it is known that water and ions follow the same path through the membrane; if, however, the membrane presents a whole spectrum of pore sizes of which only some are accessible to ions, the experimental approach becomes more involved. But although the flow of solvent is able to increase the rate of passage of a solute, the ratio between solute and solvent will be lower in the solution leaving the membrane than in that entering. Simple drag therefore cannot be used to concentrate the solute. Only if, as in Visscher's well-known fluid circuit hypothesis (Ingraham, Peters & Visscher, 1938), the pure solvent is returned by an active process, will the net effect of the solute drag be a concentration of the solute. Whether or not a mechanism of this type is operative in certain cases, it is hard to believe that it is of general importance. It is, for instance, difficult to see how a stream of water could be specific enough to carry along sodium ions without affecting potassium or chloride ions. Ruling out, then, the fluid circuit hypothesis as a general mechanism of active transport, we are left with the hypotheses that are based on chemical reactions between the ion which is being transported and the cell constituents. This interaction is usually visualized as the chemical binding to the carrier at one boundary and the release at the other boundary of the ion from the complex after some chemical alteration of the carrier. The realization that even the monovalent ions form organic complexes has somewhat lessened the objections to the carrier theory. Nevertheless, it is still based on the method of elimination, and the chemical isolation of some carrier molecule is highly desirable to complete the case.

Summing up then, for membranes where the net water transfer rate is insignificant or nil, it seems justifiable to speak of active transport (*a*) if the transfer takes place from a lower to a higher electrochemical activity and (*b*) if the transfer takes place in such a way that the flux ratio found ($M_{\text{in}} > M_{\text{out}}$) is larger than that calculated from equation (2). The number of living systems where data necessary for an analysis according to equation (2) have been obtained is still rather small. Until a few years ago the electric potential difference across most cell membranes could not be measured precisely. Recent progress in the construction and use of microelectrodes has, however, made the measurement of intracellular potentials technically possible. The mean activity coefficient of the cell interior is, on the other hand, still a matter of dispute. It is likely, and the work on isolated nerve by Keynes (1951) has indeed strengthened this belief, that the activity coefficient to be used for potassium in the cell interior can be put equal to that of the bathing fluid. We are on safer ground, however, in cases where dilute solutions of inorganic ions can be used as bathing solutions on both sides of the membrane under study. Particularly simple conditions ensue if we can use identical solutions on both sides of the membrane. Membranes useful for such an approach are the isolated frog skin and the isolated frog gastric mucosa. The experiments which I am going to discuss presently were performed on the isolated, surviving frog skin.

This organ is rather remarkable. In 1857 Du Bois-Reymond observed that it maintains a potential difference between its inside and outside. Ever since, frog skin has been one of the favourite objects of electrophysiologists and students of permeability problems. About twenty years ago another surprising property of frog skin attracted the interest of physiologists. Huf (1935) found that the isolated surviving frog skin, when in contact with Ringer's on both sides, performs an active transport of sodium chloride from the outside solution to that bathing the inside. Shortly afterwards Krogh (1937) observed that frogs in need of salt are able to take up sodium chloride from the surrounding medium, even if the latter is as dilute as 10^{-5} M with respect to sodium chloride. Even more surprising perhaps was the finding (Krogh, 1938) that the mechanism is specific to sodium. Neither potassium nor calcium were taken up at all. Among the anions Br^- and HCO_3^- were able to substitute for chloride.

If we consider the isolated skin with Ringer's on both sides, it is readily seen that the transfer of sodium must be due to active transport. Taking a skin which maintains a potential difference of 60 mV. between the inside and the outside (the former being positive relative to the latter) it follows from equation (2) that the sodium efflux ought to be ten times the influx, if the sodium diffused passively. Experiments showed, however, that the reverse is more nearly true. The influx is always higher, and sometimes more than ten times higher, than the efflux. Based on these observations the present author some years ago (Ussing, 1948) advanced the hypothesis that not only is the sodium ion actively transported, but this transport is the source of the electric potential across the skin. Obviously this hypothesis goes beyond the simple recognition of the active transport of sodium. It requires further that sodium is transferred in such a way that there is an equivalent transfer of positive electric charges. (A mechanism by which a sodium ion from the inside solution is exchanged against another positive ion from the outside solution does not fulfil this requirement.) Furthermore, it requires that no other active transport process or ion-forming metabolic processes are going on by which charges are transferred across the skin.

Just as it appears from simple inspection that the sodium ion is being actively transported, it is seen that the chloride ion might diffuse passively and that its net transfer through the skin is due to the electric potential difference. But even if the potential difference is such that it renders a passive chloride transfer thermodynamically possible, still the transfer is not necessarily truly passive. Active processes might well be aiding or resisting the diffusion of chloride. But if one measures the influx and efflux of chloride with radioactive isotopes and compares the flux ratio found with that calculated from the potential difference and the chloride concentrations (cf. equation (2)), it turns out that the agreement is quite satisfactory. Fig. 1 shows the chloride flux ratio found plotted against that calculated from a series of experiments. The efflux was determined with the radioactive ^{36}Cl, whereas the influx was determined as the sum of the efflux and the net flux (the latter value as determined by chemical analysis). The outside medium was 1/10 Ringer's, whereas the inside medium was Ringer's solution. The experiments also showed that, generally, a high potential difference was associated with a low chloride permeability and vice versa. This result is not unexpected, since the flow of chloride ions constitutes an electric current which short-circuits the skin potential more or less completely, depending on whether the resistance to chloride is low or high.

So far the observations are in agreement with the hypothesis that the potential difference is created by the active transport of sodium. However, even if the diffusion of chloride influences the skin potential only in so far as it represents a short-circuit of the electromotive force, the behaviour of the less abundant ions, notably those formed in the cell metabolism, might still contribute significantly to the potential difference observed. In particular we have to consider those metabolic ions which, like H^+ and HCO_3^-, are considered important in some hypotheses advanced to explain the skin potential.

Judged superficially, the odds against the electric asymmetry of the frog skin being the result of one transport process only seemed quite high. Nevertheless, it can be shown that, normally, the active transport of sodium ions is the sole process responsible.

Just like any electric battery the surviving frog skin with its maintained electromotive force can be short-circuited. This was demonstrated by Francis (1933), who found that the partially short-circuited frog skin would give off electric current for many hours continuously. Later, Stapp (1941) and Lund & Stapp (1947) improved the technique, using electrodes of low resistance to bring about a nearly total short-circuit of the skin. These workers did not correlate the current created with ionic movements. From the foregoing it would appear, however, that such a comparison could give important information.

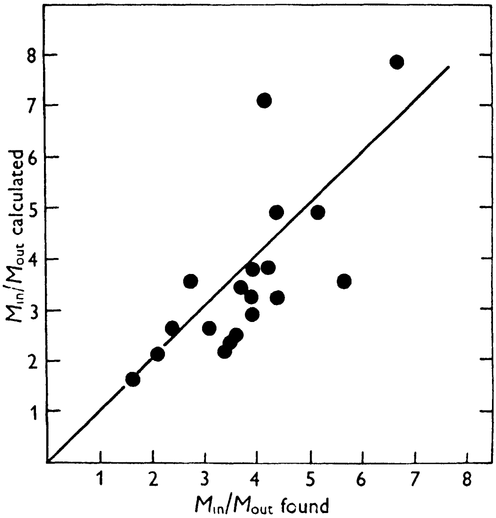

Fig. 1. Calculated versus found flux ratios (M_{in}/M_{out}) for chloride ions in the isolated surviving frog skin.

It is clear that no net transfer of passive ions can take place if the skin is short-circuited, so that the potential drop over it is nil, and if, further, the bathing solutions on the two sides are identical. Ions which are subject to active transport will, on the other hand, flow faster one way than the other, and thus contribute to the total current flowing through the short-circuit. An experimental apparatus was therefore constructed by Dr Zerahn and myself (Ussing, 1950; Ussing & Zerahn, 1951), making possible the simultaneous determination of short-circuit current and ionic fluxes. The transport rate of sodium across the skin is so low that the determination by chemical analysis of the current/active-transport relationship would meet with great difficulties. The tracer method, on the other hand, makes possible the determination of the transport rate with accuracy. The influx of sodium can be determined with ^{22}Na and the efflux with ^{24}Na (cf. Levi & Ussing, 1949), and this procedure is now being regularly used. It turned out, however, that the efflux of sodium was only a small fraction of the influx, so that it suffices in most cases to apply a suitable correction to the influx in order to obtain the net sodium transport.

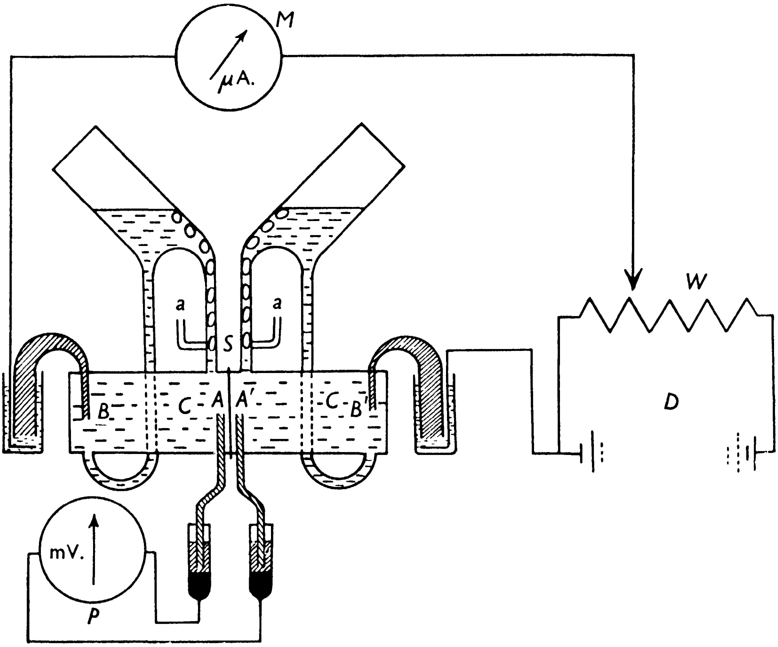

Fig. 2. Diagram of apparatus used for determining Na flux and short-circuit current. *C*, celluloid chamber containing, on each side of the skin, 40 ml. Ringer; *S*, skin; *a*, inlets for air; *A*, *A′*, agar-Ringer bridges connecting outside and inside solutions, respectively, with calomel electrodes; *B*, *B′*, agar-Ringer bridges used for applying outside e.m.f.; *D*, battery; *W*, potential divider; *M*, microammeter; *P*, tube potentiometer.

The apparatus used is shown in Fig. 2. The skin, *S*, is placed between two celluloid chambers, *C*, containing Ringer's solution. The potential differences across the skin is read on a potentiometer, *P*, which is connected through calomel electrodes to two agar-Ringer bridges, *A* and *A′*, opening close to the skin. Another pair of agar-Ringer bridges, *B* and *B′*, opening at a distance sufficient to give a homogeneous electric field at the level of the skin, are connected through silver/silver-chloride electrodes with a microammeter, *M*, and a battery, *D*. The current in this circuit is now adjusted by aid of a variable resistance, *W*, so that the potential drop across the skin is zero. It is obvious that this accomplishes a total short-circuit of the skin. The current generated is read on the microammeter. Table 1 shows the results of some of our first experiments. Influx and efflux were not determined simultaneously, but in parallel experiments. The figures are arranged so that those from influx experiments are to the left and those from the efflux experiments to the right. Both the flux values and the current are expressed as millicoulombs/cm.^{2}/hr.

Table 1. *Sodium flux and total current values obtained in* 1 *hr. periods on totally shorted normal frog skin. Group A comprises results from five influx experiments, group B results from six efflux experiments*

A (influx)			B (efflux)		
Date	millicoulomb cm.$^{-2}$hr.$^{-1}$		Date	millicoulomb cm.$^{-2}$hr.$^{-1}$	
	Na	Current		Na	Current
26. iv	102	99	28. iv	9·7	130
	93	99		10·5	139
27. iv	177	174	2. v	5·3	111
	176	162		9·1	108
	124	123		13·0	108
				13·6	112
3. v	64	63	11. v	6·0	136
	64	55		5·6	124
	57	49			
			8. vi	14·7	92
4. v	248	253		13·2	100
	260	224			
	205	205	22. ix	2·6	164
				2·4	118
23. ix	139	133			
	118	112	4. x	0·8	102

It is noticed that the efflux is always much smaller than the current, whereas the influx is identical with, or, in some cases, a little higher than, the current. On an average from a considerable number of runs, the influx is 5 % higher than the current, whereas the efflux is very nearly 5 % of the current. Thus, the net sodium flux is exactly equal to the short-circuit current. Consequently we arrive at the conclusion that the total current which can be drawn from the short-circuited frog skin comes from active sodium transport.

This holds true even if the skin is treated with agents known to affect the skin potential. Thus, 5 % carbon dioxide in the air (or oxygen) used for mixing the solutions depresses the current to zero. At the same time the sodium influx drops to a low value which, incidentally, is about equal to the efflux. This means that even the active sodium transport is stopped by 5 % carbon dioxide. This inhibition is fully reversible as can be seen from the table.

Another agent with a striking effect on the skin potential is neurohypophyseal extract which, according to Fuhrman & Ussing (1951), brings about an increase in the skin potential. The table shows that it increases the skin current as well as the influx and efflux of sodium. The net sodium flux, however, remains equal to the current.

We shall revert later to the effect of adrenaline, which is rather remarkable and constitutes the only exception so far observed to the rule of equality between current and active sodium transport. This rule has been found to hold in the presence of a long series of biologically active substances.

One may ask the question whether the mechanism responsible for the active sodium transport is specific to this ion or whether we are dealing with a more or less unspecific cation transporting system. As long as the bathing solutions are ordinary Ringer's, sodium is likely to dominate the picture compared to, say, potassium, simply due to its much higher concentration. Experiments which have recently been performed by Dr Zerahn and myself indicate, however, that the transport mechanism prefers sodium to potassium to a remarkable extent. Table 2 shows some of the results. Instead of ordinary Ringer's, bathing solutions were used where, expressed on a molar basis, 35 % of the total monovalent cation was potassium and the remaining 65 % sodium. Influx and efflux of sodium were determined simultaneously with ^{22}Na and ^{24}Na, respectively. It is seen that even under these conditions the total current is accounted for by the net sodium flux, indicating that potassium contributes insignificantly, or not at all, to the short-circuit current.

Table 2. *Showing that the current generated by the short-circuited frog skin is carried by sodium ions, even when one-third of the sodium in the bathing solutions is replaced by potassium. Area of skin, 7·1 cm.*^{2}.

	(K/Na) × 100 in solutions	Influx (μequiv. Na/hr.)	Efflux (μequiv. Na/hr.)	Na (μequiv./hr.)	Na current (μamp.)	Total current (μamp.)
I	35·0	13·2	0·58	12·6	339	289
	35·0	9·4	0·81	8·6	230	236
II	35·0	5·80	0·38	5·42	145	157
	35·0	6·13	0·35	5·78	155	154
	35·0	5·50	0·41	5·09	136	143
III	35·0	4·05	1·04	3·01	81	85
	35·0	5·30	1·06	4·24	114	116
	35·0	5·35	0·94	4·41	118	121

Experiments now in progress (Zerahn, unpublished) indicate that calcium and magnesium penetrate very slowly indeed even when present in high concentrations in the bathing solutions. The choline ion does not penetrate at all (Kirschner, in preparation).

There is, however, one ion that can to some extent substitute for sodium, namely, the lithium ion. This is clearly borne out by work which Dr Zerahn has been doing during the last year. Table 3 gives a few examples of the substitution of lithium for sodium. As bathing solutions, mixtures of ordinary Ringer's and lithium Ringer's were used. The molar ratio between lithium and sodium for the mixture used in each experiment is indicated in column 2. Influx and efflux of sodium were determined with ^{22}Na and ^{24}Na, respectively. It is seen that the net sodium current is in all cases smaller than the total short-circuit current. The part of the current not accounted for as sodium current is given in column 8 as 'lithium current'. The last column shows that this 'lithium current' comprises about the same fraction of the current as lithium does of the total monovalent cation of the bathing solutions. Furthermore, in specially designed experiments Zerahn was able to demonstrate by chemical analysis that the frog skin can transport lithium against a concentration gradient. Despite this fact lithium cannot totally substitute for sodium. In pure lithium Ringer's the skin deteriorates rapidly.

Table 3. *Showing that if part of the sodium in the solutions bathing the short-circuited frog skin is replaced by lithium, the latter ion carries a corresponding part of the current. Area of skin,* 7·1 *cm.*^{2}.

	(Li/Na) × 100 in solutions	Influx (μequiv. Na/hr.)	Efflux (μequiv. Na/hr.)	ΔNa (μequiv./hr.)	ΔNa (μamp.)	Total current (μamp.)	'Li current' (μamp.)	'Li current' % of total
I	21·2	8·5	0·7	7·7	206	284	78	27·4
	21·2	7·0	0·6	6·4	171	249	78	31·3
II	33·3	6·0	0·4	5·6	150	194	44	22·7
	33·3	3·7	0·5	3·2	86	131	45	34·3
III	52·0	2·81	0·40	2·41	65	107	42	39·2
	52·0	2·17	0·69	1·48	40	91	51	56·0
IV	80·5	0·62	0·25	1·37	37	104	67	64·0
	80·5	0·85	0·37	0·48	13	44	31	71·0

The active sodium transport of the frog skin seems to be strictly dependent upon the oxidative metabolism. Thus it is stopped by oxygen lack and by cyanide poisoning. This does not mean, however, that there is a simple relationship between the rate of sodium transport and the metabolic rate. This is clearly borne out by the fact that 5 % carbon dioxide, which inhibits the sodium transport entirely, depresses the oxygen consumption by only about 25 %.

Dr Fuhrman (1952) in our laboratory tested the effect of a number of drugs upon the sodium transport and the oxygen consumption of the frog skin. Some of the results are shown in Table 4. It is seen that, out of six drugs which inhibit sodium transport, three stimulate, two do not influence, and one inhibits the oxygen consumption. The fact that dinitrophenol inhibits the active sodium transport so strongly is perhaps an indication that ATP plays a role in the functioning of the 'sodium pump'.

Table 4. *Effect of some drugs on sodium transport and oxygen consumption of short-circuited frog skin* (*Fuhrman*)

i = inhibition; *s* = stimulation.

	Concentration drug* (M/l.)	Effect on Na transport	Effect on O_2 consumption
Dinitrophenol	5×10^{-5}	*i*	*s*
p-Nitrophenol	2×10^{-4}	*i*	*s*
Sulphanilamide	2×10^{-2}	*i*	None
p-Toluene sulphonamide	2×10^{-2}	*i*	*s*
Prontosil red	1×10^{-2}	*i*	*i*
Quinone	1×10^{-5}	*i*	None

* Concentration of inhibitor necessary to give 25–75 % inhibition of sodium transport.

Although the short-circuit current is a measure of the rate of active sodium transport across the skin it does not give any indication of the electric work performed by the living cells, which depends not only on the amount of sodium transferred, but also on the frictional resistance which has to be overcome during the passage of the ions through the skin. The tracer experiment does, however, provide the data necessary to calculate the electromotive force of the sodium transporting mechanism as well as its internal resistance. This is most easily seen if we consider an equivalent circuit representing the frog skin (see Fig. 3). E_{Na} is the electromotive force of the sodium transport mechanism; R_{Na} is the internal resistance of the 'sodium-battery', or in other words, the reciprocal of k_{Na}, that part of the total d.c. conductivity of the skin which is due to the sodium ion; $R_{\Sigma I}$ is the resistance of the shunt brought about by all the passive ions present in the skin. When in our experimental apparatus we adjust the potential

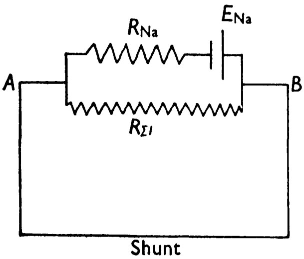

Fig. 3. Equivalent circuit representing the short-circuited frog skin. E_{Na}, electromotive force of the sodium transporting mechanism; $R_{\Sigma I}$, resistance to the Na current; R_{Na}, resistance to passive ions. The lead marked 'Shunt' represents the net effect of the applied e.m.f.

difference between the two sides of the skin to zero, the effect is that of connecting the points A and B with a shunt of infinitely high conductivity. The fraction of the current passing that shunt will therefore be infinitely larger than that passing through $R_{\Sigma I}$, which therefore becomes virtually zero. The amount of current drawn from the skin under these conditions depends only upon E_{Na} and R_{Na}, whereas the value of $R_{\Sigma I}$ is immaterial.

E_{Na} can be estimated by three independent methods. Perhaps the most obvious method is to apply a counter electromotive force and adjust it so that the sodium influx and efflux become equal. This method leads to values around 110 mV.

If E_{Na} is to be determined according to the second method, the potential is maintained at zero while the sodium concentration of the outside solution is lowered until influx and efflux become equal. The electromotive force of the 'sodium pump' pushing sodium inward then is equal to the diffusion force tending to force sodium outward. The best procedure is to replace the sodium chloride of the outside solution stepwise with the chloride of a non-penetrating monovalent ion, for example choline. In that case the ionic strengths of the inside and outside solutions remain the same and one can probably disregard the activity coefficients and put the diffusion force acting on the sodium ion equal to

$$E_{\mathrm{Na}}=\frac{RT}{F}\ln\frac{c_{\mathrm{i}}}{c_{\mathrm{o}}},$$

where c_{i} and c_{o} are the sodium concentrations in the inside and outside solutions, respectively.

The third method for determining E_{Na} depends on the assumption that the electromotive force of the 'sodium pump' affects the flux ratio of the sodium ion in exactly the same way as an applied electromotive force would affect the flux ratio of a passive ion. Thus, if we find the flux ratio 10/1 for a shorted skin we conclude that the E_{Na} responsible must be 58 mV. In general, we have that with identical solutions on both sides and zero potential difference

$$E_{\mathrm{Na}}=\frac{RT}{F}\ln\frac{M_{\mathrm{in}}}{M_{\mathrm{out}}}.$$

Since the sodium current strength is $(M_{\mathrm{in}}-M_{\mathrm{out}})$, we obtain by Ohm's law

$$R_{\mathrm{Na}}=\frac{\frac{RT}{F}\ln\frac{M_{\mathrm{in}}}{M_{\mathrm{out}}}}{M_{\mathrm{in}}-M_{\mathrm{out}}}.$$

It is seen that the tracer experiments provide the data for the estimation of both the electromotive force and the internal resistance of the 'sodium pump'. Linderholm (1952), working in Teorell's laboratory, has shown that the sodium conductivity ($1/R_{Na}$) plus the chloride conductivity ($1/R_{Cl}$) equals the d.c. conductivity of the frog skin. In the equivalent circuit (Fig. 3) we can thus put $R_{\Sigma I}$ equal to R_{Cl} which can be obtained from experiments with radioactive chloride. Thus drugs can affect the potential difference across the frog skin by affecting one or more of the three parameters E_{Na}, R_{Na} and R_{Cl}. Cu^{++} may be mentioned as an agent that increases the skin potential by increasing R_{Cl} without affecting E_{Na} and R_{Na}. Most agents affecting the skin potential do so, however, by affecting the latter two parameters or one of them. Table 5 (Kirschner, 1953) shows two examples of this. Tetraethylpyrophosphate, a potent inhibitor of cholinesterase, when added to the inside medium, depresses E_{Na} very markedly while increasing R_{Na}. Other inhibitors of cholinesterase, like eserine, have similar effects. Atropine has exactly the opposite effect, increasing the electromotive force of the 'sodium pump' while decreasing its internal resistance. These findings suggest, but do not prove, the participation of acetylcholine in the sodium transport mechanism. Anyway, it is tempting to see the active sodium transport of the frog skin as a manifestation of a general property of animal cells, which in the nerve fibre and muscle fibre serves to extrude sodium and keep up the membrane potential, but which in the frog skin, the kidney tubule and the intestinal mucosa serves the purpose of salt transport.

Table 5. *Effect of tetraethylpyrophosphate (TEPP) and atropine on active sodium transport of short-circuited skin of* Rana esculenta

Influx and efflux: μM./hr./7·07 cm.^{2}
Current: μamp./7·07 cm.^{2}
C, control period.
E, experimental period.

		E_{Na} (mV.)	R_{Na} (ohms/cm.^{2})	Flux (μM/hr.)		*I* (μamp.)
				In	Out	
I. 4×10^{-3}M-TEPP (inside)	C	35	4060	3·24	0·84	61
	E	7	(16500)	0·57	0·46	3
II. 4×10^{-3}M-TEPP (inside)	*C*	45	3030	4·93	0·84	104
	E	2	(7000)	0·90	0·82	2
I. 1×10^{-2}M-Atropine (outside)	*C*	35	7000	1·86	0·48	35
	E	67	2300	8·66	0·60	205
II. 9×10^{-3}M-Aropine (outside)	*C*	41	3800	3·62	0·78	76
	E	66	1570	12·16	1·04	297

This should not lead us to believe, however, that electric potentials across living membranes are always the result of active sodium transport. Even the frog skin may under certain conditions perform active transport of at least one more ion, namely, chloride. When the skin is stimulated by adrenaline, an extra source of electromotive force is aroused which turns out to be an active outward transport of chloride ions (Koefoed-Johnsen, Ussing & Zerahn, 1952). This transport seems to be performed by the skin glands which start secreting under the influence of adrenaline. Recently Jørgensen (unpublished) has observed that live frogs are able to perform an active transport of chloride inward when in need of this ion.

Active transport of chloride ions is also at work in the isolated frog gastric mucosa. Hogben (1951) has shown that the total electric current generated by the short-circuited gastric mucosa comes from active transport of chloride ions. Table 6 shows one of his experiments. Identical solutions were used on both sides of the mucosa and the potential was short-circuited according to the principles outlined above. Influx (secretion to nutrient side) was determined with ^{38}Cl, whereas efflux was measured with ^{36}Cl. The flux values are expressed as micro-equivalents/cm.^{2}/hr. It is seen that the net flux of chloride is 3·82. This figure equals, within the accuracy of the methods used, the sum of the electric current drawn from and the hydrochloric acid secreted by the mucosa. This is not the place to discuss the role of active chloride transport in the formation of the gastric juice, but it is evident that no explanation of the function of the gastric mucosa is complete until it takes into account the active chloride transport.

Table 6. *Chloride transfer across the short-circuited gastric mucosa of the frog*

^{36}Cl	N−S	6·80	Current	3·06
^{38}Cl	S−N	2·98	H ion	0·71
		3·82		3·77

Fluxes and current expressed as μequiv./cm.^{2}/hr. *N*, nutrient side; *S*, secretion side.

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MOVEMENTS OF CATIONS DURING RECOVERY IN NERVE

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I. INTRODUCTION

There are good reasons for believing that the conduction of impulses in excitable tissues is intimately connected with movements of sodium and potassium ions. In the giant axons of squid and cuttlefish the sequence of events is thought to be as follows. When the membrane is depolarized, either by application of a cathode or by activity in a neighbouring region of the fibre, it becomes highly and specifically permeable to Na^+ ions. As the sodium concentration is much higher outside than inside, Na^+ ions rush inwards, at first further reducing the membrane potential, and finally reversing it by some 50 mV. Near the peak of the spike, the permeability to sodium is reduced, while that to potassium is considerably increased. The potassium concentration gradient is directed outwards, so that there follows a rapid outward passage of K^+ ions, which only ceases when the membrane potential has been restored to its original value. The impulse having passed, the fibre is left with slightly more sodium inside it—and less potassium—than it had before. Evidence of this has been provided by tracer studies (Rothenberg, 1950; Grundfest & Nachmansohn, 1950; Keynes, 1951*a*, *b*) and analyses of stimulated axons (Keynes & Lewis, 1951), while the permeability changes have also been investigated in detail by electrical recording methods (Hodgkin & Katz, 1949; Hodgkin, 1951; Hodgkin, Huxley & Katz, 1952).

If the nerve is to continue to conduct impulses over long periods of time, it must possess a mechanism for pumping out sodium and for reabsorbing potassium. Evidence for potassium reabsorption is provided by the experiments of Shanes (1951), but little is known about the fate of the sodium which enters nerve and muscle fibres during electrical activity. In contrast to the conduction mechanism, where ions move down pre-existing concentration gradients, the recovery process necessitates the performance of secretory work, since the ions are transported from weak to stronger solutions. We have recently been using radioactive tracer techniques to study this active transport of sodium and potassium across nerve and muscle membranes, and shall present here some of our preliminary findings.

II. THE NATURE OF THE SODIUM EFFLUX IN NERVE AND MUSCLE

If giant cephalopod axons or frog muscles are loaded with ^{24}Na by stimulation or prolonged soaking in radioactive solutions, and are then washed in a rapid stream of inactive artificial sea water or Ringer, their radioactivity is found to decrease in a roughly exponential manner (Levi & Ussing, 1948; Harris & Burn, 1949; Keynes, 1951*b*). There is, therefore, a continual movement of sodium outwards through the cell membrane. This sodium efflux can conveniently be investigated experimentally, but we must first inquire whether it can legitimately be identified with the operation of a secretory mechanism, or whether it should more properly be regarded merely as a passive diffusion along the concentration and potential gradients. One test is to apply the equation derived by Ussing (1949*a*) and Teorell (1949) relating the ratio of the inward and outward ionic fluxes to the electrochemical activities of the ions on either side of the membrane. It has been shown that for the independent diffusion of free ions,

$$\frac{M_{\text{inwards}}}{M_{\text{outwards}}}=\frac{f_o}{f_i}\frac{C_o}{C_i}e^{EF/RT}, \tag{1}$$

where the M's represent the fluxes of a given ion, f_o and f_i are its activity coefficients, C_o and C_i the external and internal concentrations, and E is the potential difference between the external solution and the axoplasm. There is no *a priori* reason for assuming that sodium ions do cross the membrane independently, but electrical studies suggest that equation (1) may be valid, at least approximately, for the rapid sodium movements which occur during a nervous impulse (Hodgkin & Huxley, 1952). The first measurements of the sodium fluxes in 200μ *Sepia* axons (Keynes, 1951*b*) gave an influx of 61 and an efflux of 31 p.mol./cm.^{2}/sec., but in most of our recent experiments the fluxes have been nearly equal, averaging about 40 p.mol./cm.^{2}/sec. for internal sodium concentrations generally between 40 and 100 mmol./l. axoplasm. These axons had all been stimulated for about 10 min. at 50 impulses/sec. before making any measurements, in order to ensure that the recovery process was working under approximately standard conditions. On the whole, they were probably in somewhat better condition than the axons used in the original work, being longer and dissected by improved methods, and this is likely to explain their lower sodium influx. In three axons similarly recovering from stimulation, the resting potential determined with internal microelectrodes was 60–70 mV.; Weidmann (1951) found 62 mV. in some unstimulated axons. Assuming equal activity coefficients in the axoplasm and in sea water, and taking C_0 as 485 mM, the theoretical flux ratio calculated from equation (1) lies between 200:1 and 50:1, as compared with the observed ratio close to unity. It follows that although from 0·5 to 2% of the outward sodium flux can reasonably be ascribed to a purely passive diffusion process, some other explanation must be found for the remainder. For frog muscles the conclusion is similar, since the figures given by Hodgkin (1951) lead to a theoretical ratio of over 150/1.

If the amount of energy required to extrude sodium at the observed rate were greater than the resting metabolism of the tissue could possibly provide, it would be justifiable to conclude that only part of the sodium efflux could be an active secretion. Levi & Ussing (1948) calculated that about 30% of the resting metabolism of a frog sartorius muscle would be needed for sodium extrusion—a figure which seemed to demand a rather high efficiency for the sodium pump. However, there is some indication that their estimate may have been too large, since some more recent experiments in which the extrusion of ^{24}Na from pairs of frog sartorii was measured in parallel with their oxygen consumption gave an average energy requirement of only 10% (Keynes & Marshall, 1954). Furthermore, in *Sepia* axons the energy used for sodium extrusion would not constitute an immoderate proportion of the resting metabolism. It can be calculated that sodium is moved outwards at a rate of roughly 3×10^{-5} mol./g. axoplasm/hr., against a total electrochemical potential difference of the order of 115 mV. This would need about 0·08 cal./g. axoplasm/hr. of secretory work, which represents some 10% of the resting oxygen consumption. (Cardot, Faure & Arvanitaki (1950) found that isolated *Sepia* axons consumed 1·6 cu.mm. O_2/mg. dry weight/hr. when soaked in sea water.) Thus in neither tissue do energy considerations rule out the possibility that the whole of the sodium efflux may represent an active secretion.*

An alternative to active secretion of sodium has been suggested by Ussing (1949*b*), who pointed out that sodium might conceivably be exchanged across the membrane without the performance of any osmotic work, if its inward and outward movements were suitably linked. Such a mechanism would not, of course, bring about any net transfer of sodium. We cannot be certain that this idea is wholly inapplicable to *Sepia* axons, but observations of their behaviour in sodium-free solutions show that it will not provide a complete explanation of the observed sodium efflux. When axons containing ^{24}Na are transferred from inactive artificial sea

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* An interesting way of expressing the relationship between total oxygen consumption and sodium movement is to give the number of ions transferred for each molecule of oxygen consumed (cf. Davies, this Symposium). The figures used here indicate a ratio of four Na^+ ions per O_2 in both *Sepia* axons and frog muscle.

water to a solution in which the sodium is completely replaced by either choline or dextrose, the immediate effect is an increase in sodium efflux of about 30 %. This seems inconsistent with an obligatory coupling between influx and efflux of the type postulated by Ussing.

Another reason for thinking that the sodium efflux observed with tracers does represent a secretory extrusion is that fluxes of the same order can be calculated from the net movements of sodium which have been observed in frog muscle. Following Steinbach (1951, 1952), Desmedt (1953) measured the rate at which frog muscles can pump out sodium when they are taken from a potassium-deficient medium in which their intracellular sodium concentration has become abnormally high, to a potassium-rich solution in which they extrude some of the sodium against the concentration gradient. From the curves given by Desmedt it can be calculated that the maximum rate of net outward sodium movement is about 20 p.mol./cm.^{2}/sec., which is not very different from the sodium efflux found by tracer methods (Keynes, 1954).

It is difficult to decide how much importance should be attached to the suggestion that part of the observed sodium efflux arises from an exchange of sodium bound in some way on the outer side of the membrane, and does not represent a passage of ions through the membrane (Harris, 1950). In squid axons most of the ^{24}Na appears to be intracellular, since it can be extruded with the axoplasm. In muscle, the size of the overshoot of the action potential under various conditions is consistent with the assumption that all the sodium associated with the fibre space is intracellular (Desmedt, 1953). We shall therefore take it that there is not enough externally bound sodium to cause material disturbance to our arguments.

III. METHODS OF MEASURING IONIC FLUXES

Two types of technique have been used to measure the ionic fluxes in giant cephalopod axons. One consists in measuring the radioactivity of the axon itself, immersed in a rapidly flowing inactive medium, using the apparatus described by Keynes (1951*b*). Influxes are obtained from the amount of radioactivity found to enter the axoplasm during a short soak in a labelled solution, while effluxes are calculated from the rate at which the count decreases over a relatively long period in the inactive artificial sea water. This method serves well for determining influxes, but is not really suitable for observing small changes in the ionic effluxes. For this purpose it can easily be shown that the reliability of the results is greatly improved by measuring the radioactivity which appears in the external medium, instead of that which remains inside the axon, the gain in accuracy being most marked when the efflux is smallest. Our second technique is illustrated in Figs. 1 and 2, which show two forms of the apparatus used for slightly different purposes. The axons were first loaded with radioactivity by stimulation in ^{24}Na or by soaking in ^{42}K artificial sea water, and were then pulled through a short length of precision-bore capillary tubing. For experiments with inhibitors (Fig. 1) fluid was withdrawn from a single side-arm, so that all the radioactive ions extruded from the central 30 mm. of the axon were collected; end-effects were minimized by continually changing the fluid in the open side puddles. The radioactivity of 5 ml.

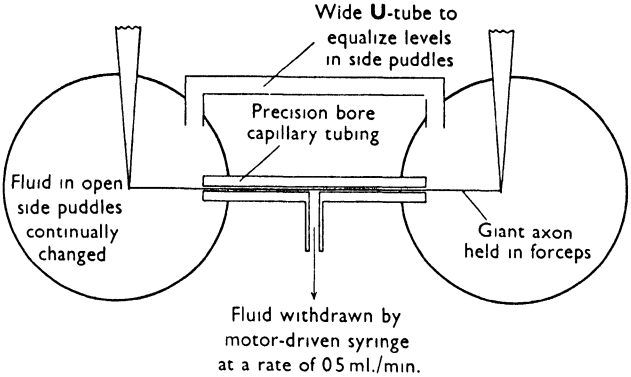

Fig. 1. Apparatus (not to scale) for collecting radioactive ions emerging from giant cephalopod axons. The overall length of the capillary was 30 mm. For 500 μ squid axons the internal diameter of the tubing was 1 mm.; for 200 μ *Sepia* axons it was 600 μ.

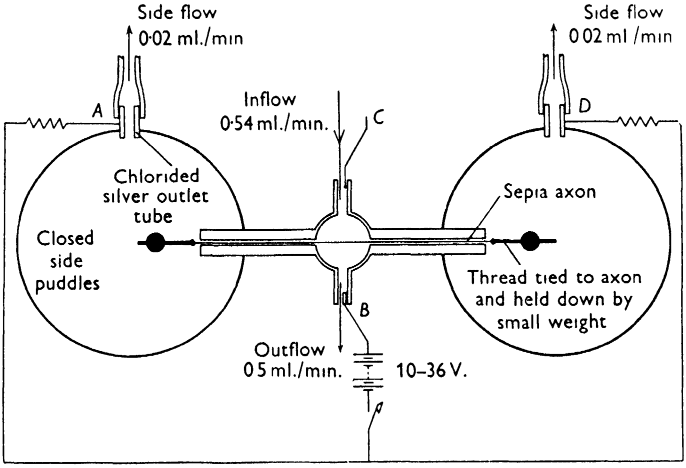

Fig. 2. Apparatus (not to scale) for collecting ^{24}Na extruded from *Sepia* axons during application of a polarizing current. The central bulb was 6 mm. across, and the length of capillary tubing on either side was 20 mm. The internal diameter of the capillary was 375 μ. *A* and *D* were silver tubes, chlorided on the inside. *B* was a large chlorided silver wire, and *C* a small one.

samples was measured in a conventional liquid counter. For other experiments, in which membrane potential was one of the variables, radioactive ions were only collected from a central 6 mm. bulb across which artificial sea water flowed (Fig. 2). ^{24}Na emerging from the part of the axon in the capillary was prevented from reaching the bulb by ensuring that about 10% of the washing solution flowed sideways into the sealed side puddles, from which fluid was sucked very slowly by a pair of motor-driven syringes. The membrane potential of the 6 mm. central stretch of the axon could be raised fairly uniformly by applying a polarizing voltage between the bulb and the two side puddles. Again the radioactivity of 5 ml. samples collected from the bulb was determined with a liquid counter.

On every occasion when the absolute size of the sodium or potassium efflux was to be determined, it was essential to know the total concentrations of sodium and potassium in the axoplasm. At the end of each successful experiment on a *Sepia* axon, a known length of axon was cut out and dried on a quartz thread, for subsequent determination of sodium and potassium by activation analysis (Keynes & Lewis, 1951); concentrations were calculated from the length of axon excised and the mean axon diameter. In squid experiments, a sample of axoplasm was extruded on to a quartz hook, weighed with a torsion balance, dried, and stored in a quartz tube for analysis.

IV. THE EFFECT OF METABOLIC INHIBITORS ON THE SODIUM FLUXES

Fig. 3 shows the results of an experiment on the sodium efflux from a *Sepia* axon, using the method of Fig. 1. In normal artificial sea water the rate of appearance of ^{24}Na in the external medium (expressed in counts/min./min.) declines exponentially, because the intracellular ^{24}Na is being diluted all the time with inactive sodium; nevertheless, the absolute size of the sodium efflux is probably nearly constant. When 0·2 mM-dinitrophenol (DNP) is added to the washing fluid, the efflux decreases gradually to about one-twentieth of its initial value. The effect is largely reversed by washing the DNP away, when the efflux soon recovers to a level comparable with that before inhibition. The older technique gives an equivalent result, the ^{24}Na content of a poisoned axon remaining so nearly constant over a period of an hour or so that it is hard to obtain a reliable figure for the very small residual sodium efflux. Two other metabolic inhibitors, cyanide in concentrations of 1 and 10 mM and 3 mM-azide, have been found to have a very similar effect; a single experiment with 10 mM cyanide, performed some time ago with the original method, showed no effect (Keynes, 1951*c*), presumably because the inhibitor was not applied for long enough.

The action of DNP on the sodium efflux persists under all conditions so far tested. Thus the efflux remains at a very low value when choline is substituted for sodium in the external medium, and even when the axon is transferred to an isotonic dextrose solution containing no salts other than the 0·2 mM of DNP. The variation in sodium influx during treatment with DNP has also been examined. As the figures in Table 1 show, there is some reduction in influx in a fully poisoned axon, but not by much more than half.

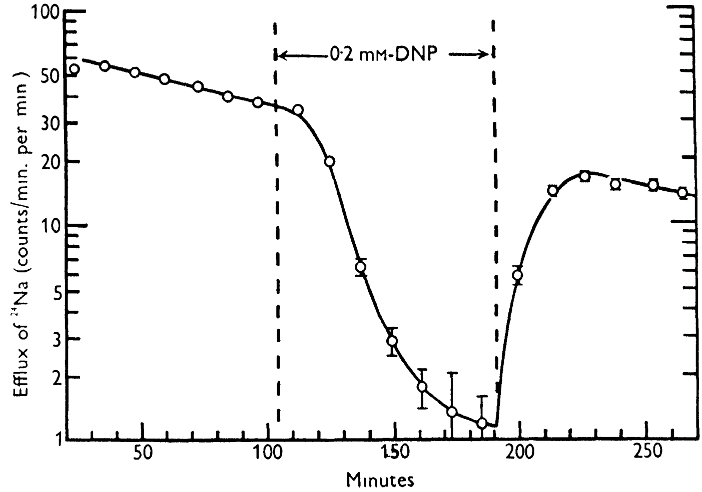

Fig. 3. Sodium efflux from a *Sepia* axon during treatment with 0·2 mM-dinitrophenol. At beginning and end of experiment axon was in normal artificial sea water. Abscissa: time after end of stimulation in radioactive solution. Ordinate: rate at which ^{24}Na leaves axon. Vertical lines are ±2 × S.E. (From Hodgkin & Keynes, 1953*a*.)

This interruption of the sodium efflux in cephalopod axons is by no means the only example of the action of metabolic inhibitors on the active secretion of ions. Poisons like cyanide and DNP have been shown to block ionic transport in a variety of animal tissues, such as gastric mucosa (Davies, 1951), kidney slices (Mudge, 1951), frog skin (Fuhrman, 1952) and chicken erythrocytes (Maizels, 1954), as well as in plants (Robertson, Wilkins, & Weeks, 1951). However, in frog muscle inhibitors do not seem to have a very pronounced effect on the sodium efflux, although it is difficult to be certain that they do not cause some reduction. Neither 0·2 mM-DNP, nor a combination of 3 mM cyanide with 0·5 mM iodoacetate, applied to sartorius muscles for over 3 hr. at 20° C., caused any large reduction in the rate of loss of ^{24}Na in inactive Ringer (Keynes & Marshall, 1954).

V. THE EXCITABILITY OF POISONED AXONS

The marked reduction in the sodium efflux of a poisoned axon is not accompanied by loss of excitability. On one occasion a *Sepia* axon 180μ in diameter, treated with DNP, conducted impulses for 70 min. at a frequency of 50/sec.—a performance comparable with that expected of a normal fibre. This is not surprising, because the net rate of gain of sodium at 50 impulses/sec. is fairly high, and it makes little difference to the internal sodium concentration whether the pump is working or not. Experiments with external electrodes in *Sepia* axons and with long internal electrodes in squid axons have shown that the action potential and resting potential are almost unaltered by DNP. Similar but less complete results have been obtained with azide and cyanide. A typical experiment with 0·2 mM-DNP on a squid axon is illustrated in Fig. 4. The only noticeable effects of the inhibitor were (1) a slight acceleration in the rate of decline of the spike, such as might occur if sodium were slowly accumulating inside the axon, and (2) an initial rise in the resting potential of about 2 mV., followed by a very slow decline. After about 1 hr. in DNP the resting potential and action potential were almost exactly equal to the means of the values before and after treatment. In these experiments the axons were surrounded by a relatively large volume of sea water, and it is possible that accumulation of potassium in the external fluid might have caused inexcitability had the

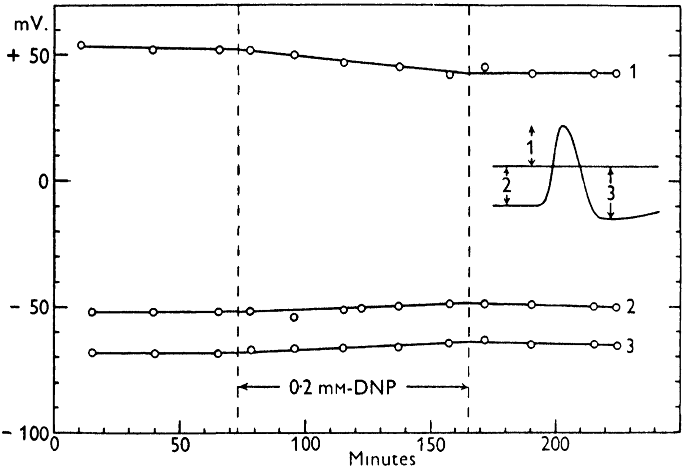

Fig. 4. Effect of dinitrophenol on membrane potentials in a squid axon. The potential of an internal electrode relative to that of the external solution was determined at rest (2), at crest of spike (1), and at maximum of positive phase (3). At beginning and end of experiment axon was in normal artificial sea water.

volume been much smaller (see the work of Shanes & Hopkins (1948) and Shanes (1951) on the effect of anoxia on crustacean and squid nerve).

It is not unreasonable to find that inhibitors do not interfere with the excitability of giant axons, because there is already other evidence that the immediate source of energy for the transmission of the nerve impulse is not metabolic, but is derived from the movement of ions down the concentration gradients. In order to test this point further, we have used ^{24}Na to measure the rapid sodium movements during stimulation of poisoned fibres. At the beginning of one experiment, the result of 4 min. stimulation (at 50 impulses/sec.) of a squid axon in artificial sea water made up with ^{24}Na was to cause an extra sodium entry of 11·5 p.mol./cm.^{2}/impulse; the resting influx was 50 p.mol./cm.^{2}/sec. The axon was then treated with 0·2 mM-DNP for an hour, reducing the sodium efflux almost to zero as usual. Stimulation in a ^{24}Na solution containing 0·2 mM-DNP now caused a sodium entry of 10·7 p.mol./cm.^{2}/impulse (assuming the resting sodium influx to have been unaltered by DNP; if the influx were actually halved, the correct result would be slightly greater—11·1 p.mol./cm.^{2}/impulse). When the axon had been allowed to recover in normal artificial sea water for an hour, a final period of stimulation gave an entry of 11·9 p.mol./cm.^{2}/impulse. In another experiment, the extra outward movement of sodium was measured during electrical activity, using the technique shown in Fig. 1. In normal artificial sea water at the start and finish, stimulation gave rise to outward sodium movements estimated as 5·9 and 5·4 p.mol./cm.^{2}/impulse. In 0·2 mM-DNP, with the resting efflux reduced to about one-tenth, stimulation still resulted in an outward sodium movement of 4·6 p.mol./cm.^{2}/impulse.

At least in cephalopod axons, it thus seems that the mechanism responsible for the conduction of impulses can be dissociated from that which restores the ionic concentration differences after activity. This conclusion is not necessarily valid for other kinds of excitable tissue. Interpretation of the results of treating frog muscle (Ling & Gerard, 1949), frog nerve (Lorente de Nó, 1947) and crustacean nerve (Shanes & Hopkins, 1948) with inhibitors, or of depriving them of oxygen, is complicated by the possibility that some of the observed effects may arise secondarily from changes in internal ionic concentrations together with an accumulation of potassium outside the fibres. However, there are definite indications that in some cases metabolic poisons may have a direct influence on the membrane potential.

VI. THE EFFECT OF EXTERNAL POTASSIUM ON THE SODIUM EFFLUX

Steinbach (1940, 1951, 1952) has shown that when frog muscles are soaked in potassium-free Ringer's solution they lose potassium and gain sodium, and that if they are then transferred to potassium-rich Ringer they are able to extrude some of their surplus internal sodium. Part of the explanation for this behaviour appears to be that the sodium efflux is appreciably decreased in potassium-free Ringer, and is increased well above normal in potassium-rich Ringer. The changes are most easily observed in a very small muscle like the extensor longus dig. IV; in the sartorius they tend to be obscured by diffusion effects (Keynes, 1954).

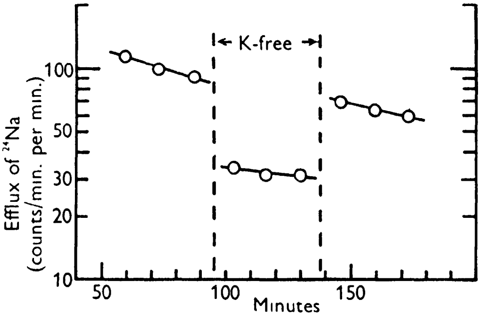

Fig. 5. Effect of a potassium-free solution on sodium efflux from a *Sepia* axon. At the beginning and end of the experiment the axon was in normal artificial sea water, in which potassium concentration was 10·35 mM. Abscissa: time after end of stimulation in radioactive solution. Ordinate: rate at which ^{24}Na leaves axon. (From Hodgkin & Keynes, 1953*b*.)

A similar reduction of sodium efflux in a potassium-free medium has been noted in human erythrocytes by Harris & Maizels (1951). As Fig. 5 shows, *Sepia* axons behave in the same way. In potassium-free artificial sea water the sodium efflux is reversibly reduced to about one-third of its normal value, the effect apparently being immediate, in contrast to the delayed action of inhibitors. High potassium concentrations cause an increase in sodium efflux, but not a very large one, the efflux only being raised to 30% above normal when the external potassium concentration is 50 mM.

It seemed of some interest to inquire into the reason for this effect. In the first place, we have found that it cannot be due to a decreased sodium permeability in both directions, since the sodium influx is not significantly different from normal in potassium-free artificial sea water (see figures in Table 1). Another explanation might be that there is a rise in resting potential when the external potassium is removed, and that this slows sodium extrusion by increasing the electrical potential gradient against which a positively charged ion has to be ejected. The effect of potassium concentration on the resting potential in *Sepia* axons has been determined with internal microelectrodes, and the rise in potassium-free sea water turns out to be 5–10 mV. Other types of nerve fibre give changes of the same order (see Hodgkin, 1951).

In order to find out whether an increase of 10 mV. in membrane potential could reduce the sodium efflux enough to account for the effect of removing external potassium, we used the apparatus shown in Fig. 2. This was so designed that the efflux from a 6 mm. length of *Sepia* axon could be measured while the membrane potential was varied. Before starting an experiment, current was applied between *A* and *B*, and the electrotonic potential it produced was measured between *C* and *D*. A similar measurement was made at the end of each group of determinations, in order to allow for changes in membrane resistance. From these readings it was possible to estimate the average change in membrane potential produced when current was applied both to *A* and *D* as in Fig. 2. The results of a typical experiment are shown in Fig. 6. It will be seen that with polarising currents which gave mean potential increases of 17 and 28 mV., there was no significant alteration in the rate at which sodium left the axon, although

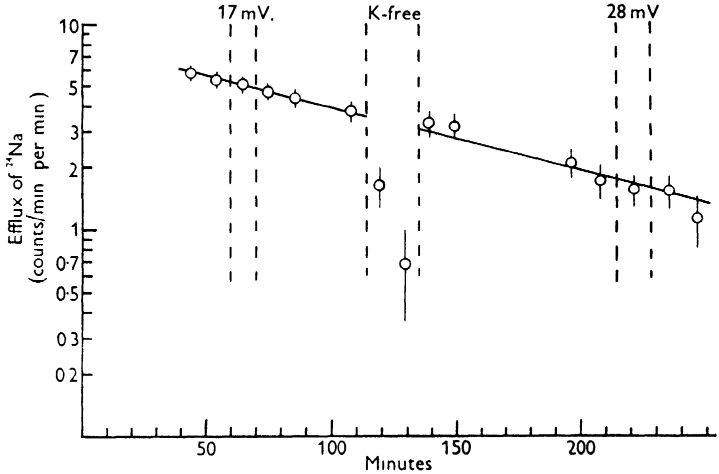

Fig. 6. Effect of anodal polarization and a potassium-free solution on sodium efflux from a *Sepia* axon. Abscissa: time in minutes. Ordinate: rate at which ^{24}Na leaves axon. Vertical lines are ±2 × S.E. The figures of 17 and 28 mV. are based on pairs of measurements giving 15 and 19 mV. in one case, and 36 and 20 mV. in the other. The apparent lag in the effect of the potassium-free solution is explained by the time taken to change solutions, the apparatus not being washed out between samples as it was in the experiments of Figs. 3 and 5.

a potassium-free solution caused a substantial reduction. Similar results were obtained in other experiments, the average change in efflux being 0·99 ± 0·04 (S.E. of mean) for an increase in membrane potential averaging 18 mV. This indicates that the effect of removing external potassium cannot be due to its action in increasing membrane potential.

VII. COUPLING BETWEEN POTASSIUM INFLUX AND SODIUM EFFLUX

Another possibility is that there may be some more specific form of linkage between potassium influx and sodium efflux, of the type proposed for erythrocytes by Harris & Maizels (1952). Thus one might imagine a cyclical mechanism in which a potassium carrier (X) moved inwards in association with potassium, was converted by metabolism into a sodium carrier (Y) on the inside of the membrane, and returned to the outside in association with sodium. A further conversion of Y into X on the outside of the membrane would complete the cycle, and sodium would move outwards on one limb while potassium moved inwards on the other. A system of this kind would be inhibited both by removal of external potassium and by interfering with the metabolic activity of the cell. Support for this type of hypothesis is provided by the action of DNP and cyanide on the potassium influx of *Sepia* fibres recovering from a bout of stimulation. As Table 1 shows, when these inhibitors were applied in concentrations sufficient to cause a drastic reduction in sodium efflux, there was a reversible reduction in potassium influx to about one seventh of the normal value. This was not due to a reduction in the potassium permeability of the membrane, since the potassium efflux was, if anything, somewhat increased by 0·2 mM-DNP or cyanide. Nor was it due to a change in membrane potential, since we have shown that this is virtually unaltered by DNP. The most reasonable explanation is that, in addition to moving passively through the membrane, potassium ions may also be drawn into the cell by a metabolic process coupled to one which simultaneously extrudes sodium.

The idea of a coupling between potassium influx and sodium efflux is attractive because it would explain both the action of inhibitors on potassium influx and the effect of external potassium on sodium efflux. It also seems consistent with the observations that inhibition of the sodium pump has little effect on the resting potential, and that alterations of membrane potential do not change the sodium efflux. Thus if sodium extrusion were coupled to potassium absorption, the secretory process would transfer no charge across the membrane, so that it might be expected neither to affect the membrane potential directly, nor to be altered by changes in membrane potential. On the other hand, the hypothesis raises a number of difficulties which have not yet been resolved. In the first place it is clear that the link between potassium influx and sodium efflux is not at all rigid, since the latter is found to continue at about its normal rate (or somewhat above it) when all ions are removed from the external medium and tonicity is maintained with dextrose. Under these conditions sodium is no longer moving against a concentration gradient, so that the situation is hardly comparable with the normal one. Nevertheless, it seems clear that the sodium efflux into sugar solutions does not involve a passive movement, since it is still blocked by DNP. We have checked that there is no detectable pH change when sodium is extruded into a small volume of isotonic dextrose, so that an exchange of Na^+ and H^+ ions can be eliminated. The only remaining alternative is that under certain conditions sodium may move out of the axon with some unidentified anion.

Table 1. *The effects of various solutions on the sodium and potassium fluxes in* Sepia *axons*

	Normal fluxes (p.mol./(cm.²/sec.)	Flux ratios in abnormal media							
		Dinitro-phenol		Cyanide			Azide 3 mM	[K] = 0 mM	[K] = 50 mM
		0·1 mM	0·2 mM	1 mM	2 mM	10 mM			
Na influx	35	—	0·5	—	—	—	—	0·9	—
Na efflux	40	0·1	0·05	0·06	—	< 0·08	< 0·08	0·3	1·3
K influx	20	—	0·13	—	0·3	—	—	0	6
K efflux	30	—	c. 1·5	—	c. 1·1	—	—	1	5

All these figures were obtained with axons which had been stimulated for about 10 min. at 50 impulses/sec. before making any measurements. The first column gives the average fluxes in a normal medium (artificial sea water). 1 p.mol. = 1 $\mu\mu$mol. = 10^{-12} mole. The other columns show the ratios of the fluxes in abnormal media to the geometric mean of the normal fluxes determined before and after treatment with the abnormal solution. Some of the figures are based on few experiments, and are subject to revision.

Another difficulty raised by our experiments is that the evidence for a secretory potassium influx destroys the apparent agreement between the observed flux ratios for potassium and those calculated for independent transport from equation (1). In the axons used in the present experiments, the potassium influx averaged just under 20 p.mol./cm.²/sec., and was usually between one-third and two-thirds of the efflux. As has been argued previously (Keynes, 1951*b*; Hodgkin, 1951), this ratio is approximately what one would expect if K^+ ions were free to diffuse independently across the membrane. It is now clear that the agreement must be a coincidence, since a large fraction of the influx depends on metabolism, and the flux ratio falls to 0·05 in poisoned axons without appreciable changes in resting potential or potassium concentration. It also follows that even in a poisoned axon the movements of potassium ions across the membrane are not independent in the sense required for equation (1) to hold.

The evidence for an active uptake of potassium in fibres recovering from stimulation has been emphasized because it was an unexpected result. However, it should not be thought that potassium can only move into nerve fibres through a secretory channel. If axons are depolarized by raising the external potassium concentration to 50 mM, the membrane becomes much more permeable to potassium, and permits large fluxes of potassium to pass in both directions. Under these conditions we have found that DNP apparently has very little effect, since the influx and efflux in poisoned fibres are close to the average values for unpoisoned fibres in 50 mM-potassium (see Table 1). This is satisfactory, because there is other evidence, both from electrical studies (Hodgkin & Huxley, 1952) and from tracer experiments (Hodgkin & Huxley, 1953), that depolarization causes a maintained increase in the potassium conductance of the nerve membrane, and that this change has an important role in the conduction of impulses. It therefore seems likely that potassium can cross the membrane by two parallel routes—a secretory channel and a passive permeability channel. But there is clearly not yet enough evidence to apportion the normal fluxes between these two pathways.

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THE REGULATION OF SODIUM AND POTASSIUM IN MUSCLE FIBRES

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I. INTRODUCTION

One of the first manifestations of life must have been a differentiation of living forms from the environment. In basic chemical make-up there is a remarkable uniformity found in all types of cells that have been investigated. This uniformity appears to extend even to such stable, diffusible substances as the inorganic ion-forming metals, sodium and potassium. The ratio of internal to external concentration is always higher for potassium than for sodium; usually the absolute internal concentration of potassium is higher than that of sodium. Since these elements are rather similar with respect to their physical and chemical properties, and since all normal active cells are known to be permeable to them, it follows that mechanisms for selective elimination or uptake of the two must have developed at an early period in the evolution of life.

Leaving, for the moment, single cells, and turning to higher multicellular forms, for example, the frog or the crab, an equally remarkable ability to regulate the internal body fluids with respect to ions is found. Oddly enough, the regulation of the body fluids with respect to sodium and potassium is the precise opposite of that of the individual cells. As is well known, many salt-water Crustacea can tolerate considerable dilution of sea water in which they live with only minor variations in the salt content of the body fluids. As the external medium is diluted, sodium and chloride of the blood drops slightly to a new level which is then maintained or regulated at a constant value in the face of further external dilution (cf. Krogh, 1939). On the other hand, if potassium of the body fluid is measured it is found to vary nearly directly with the external ionic strength. If tissues, such as muscle and nerve, are assayed in parallel experiments, potassium tissue concentration is found to be closely regulated; sodium tissue concentration varies as the external medium within wide limits (Steinbach, unpublished).

Thus the cells of higher animals maintain a relatively constant internal potassium concentration, yet these same cells, organized as tissues of the skin, alimentary tract and excretory tract, serve as devices for regulating the sodium content of the body fluid. It is perhaps noteworthy that, while individual plant cells appear to show the same sort of chemical differentiation with respect to sodium and potassium as animal cells do, the whole organisms have not utilized their ion-transporting devices to give the highly regulated body fluids (high sodium) shown by their more mobile animal relatives.

During normal resting existence, cells typically maintain a high-potassium, low-sodium composition of the intracellular fluids. Whole organisms, among the higher animals, also maintain a high-sodium, low-potassium body fluid. In general, there are two common categories of conditions under which both cells and whole animals tend to lose their abilities for maintaining characteristic ionic distributions: (*a*) any violent alteration tending to lead to the death of the living unit (shock, extreme temperature changes, etc.); (*b*) transitory responses to stimulation. Living units, during a response to a stimulus or during 'death changes', lose potassium from the cells and gain sodium, and the body fluids correspondingly tend to equilibrate with the external environment.

Thus, not only is the ionic differentiation a characteristic of the living organism, but it is completely dependent upon the metabolic processes that contribute to the state we call 'living'. It is towards an explanation of this delicate criterion of life that this paper is directed.

II. THE CASE OF THE ISOLATED FROG SARTORIUS MUSCLE

Of all the cell types that have been studied, probably the isolated striated muscle of the frog is the best known, barring, of course, the ubiquitous erythrocyte with all its special complications. The frog sartorius appears to be a good choice of material in general, since the distribution and movement of sodium and potassium in it appears to be quite comparable to similar phenomena in a variety of muscles from many different types of organism, vertebrate and invertebrate (cf. Steinbach, 1947). In addition, it is almost indestructible; even biochemists and physiologists can excise it with a minimum of damage, and a great many physical facts are known about its resting and contracted state.

Over a period of many years, a variety of explanations have been offered for the ability of muscle fibres to maintain a high-potassium, low-sodium condition. These explanations fall into three categories.

(1) It is assumed that muscle cells are either impermeable to all cations or to the special cation, sodium, or to cations of hydrated volume similar to sodium or greater.

(2) It is assumed that some constituents, presumably organic, within the muscle fibre can effectively bind a considerable fraction of the potassium in preference to sodium.

(3) It is assumed that the high internal potassium concentration is maintained by an electrochemical gradient, sodium entering the fibre but being transported outward by a sodium extrusion system.

The first two of these assumptions will be examined briefly, the third in more detail.

(1) *Permeability* v. *Impermeability*

Since the work of Fenn (cf. Fenn, 1936, for references) it has been known that sartorii, isolated into the usual Ringer's fluid (potassium concentration *c.* 0·002 M) tend to lose potassium and gain sodium. Except for a short interval immediately after isolation, only slight changes in concentration of inorganic anions such as chloride were observed, hence it was concluded that sodium entered the muscle fibres in exchange for potassium. Earlier, Meigs (Meigs & Atwood, 1916) had shown by many good chemical analyses that muscles treated with solutions containing great excesses of potassium would swell markedly, and that this swelling was reversible under certain conditions involving a reversible gain and loss of both potassium and chloride.

Thus, for a long time, the evidence has been very good that muscle fibres are permeable, in the strict sense of the word, to sodium, potassium and chloride. Recent work with isotopic tracers (reviewed elsewhere in this volume) has served to demonstrate this point with great clarity and to give us some rather precise numbers to work with, with respect to relative rates of penetration of the substances in question.

Permeabilities expressed in dimensions comparable to diffusion rates are of prime importance for processes that are highly time-dependent. Reversible changes during excitation, occuring in milliseconds, might well be controlled by relative rates of penetration of specific ions. Even the slower changes during growth and differentiation might depend in their details upon permeability rates. However, the steady-state changes which are reflected in the normal maintenance of the internal sodium and potassium concentration of muscle fibres are probably best regarded as relatively time-independent and hence permeabilities, as rates, will be invoked only incidentally in the present discussion.

Teorell (1949) has used a special term of some interest. In describing cases where, in the gross distribution of a substance, cells behave as though they were impermeable (i.e. the substance does not seem to enter to cause net changes in concentration), he speaks of 'false impermeability'. The frog sartorius would thus show 'false impermeability' to sodium, the erythrocyte would show 'false impermeability' to cations in general. In such instances we may be aware of a false impermeability, but in calculations of various electrochemical equilibria it is frequently useful to assume true impermeability. However, the usefulness of this assumption should not blind us to the fact that the assumption is wrong in its fundamental details.

(2) *Ion binding*

In order to explain the distribution of sodium and potassium in the frog muscle, ion binding is almost as attractive an assumption as is selective impermeability. It is, however, very difficult to discuss on a precise basis because of the lack of good evidence. Certain it is that whole muscles behave as though they contained, under certain conditions, 'quotas' of potassium which can leave the tissues with varying degrees of ease. A number of abstracts and short papers have been published claiming that myosin (the major protein constituent of muscle) and haemoglobin (occupying that same quantitative position in the erythrocyte) can selectively bind appreciable potassium in preference to sodium and thus contribute to the general ionic distribution (cf. Stone & Shapiro, 1948; Steinbach, 1950). However, studies in which pure haemoglobin (Battley & Klotz, 1951) and muscle particulate matter (Steinbach, 1950) were analysed gave no evidence for a selective binding amounting to more than a small percentage at most. In the case of the muscle tissue, any selective binding was in the direction of excess sodium rather than potassium. Recently, good evidence has been brought forth for a non-exchangeable fraction of potassium in liver mitochondria held under aerobic conditions. However, even here, only a very small fraction of the total base-binding capacity of the mitochondria is involved (cf. discussion of paper by Mudge, 1953).

That there must be some selective combination of cell constituents with both sodium and potassium is indicated by the activating and inhibiting effects of these ions on certain enzyme systems. However, here again, the amounts involved are very small as compared with the total numbers of ions present in the protoplasmic system.

Lastly, it is worth reiterating that all studies of osmotic concentration and electrical conductivity of the internal protoplasm of muscle fibres are consistent with the idea that a major fraction of the internal sodium and potassium is present as freely dissociable ions, exerting osmotic pressure and carrying electric current as in normal solutions.

Ion binding then must be discounted as a factor leading directly to excessive analytical concentrations of potassium in protoplasm in preference to sodium. However, the small bindings that have been indicated may very well be of crucial importance in active transport mechanisms.

(3) *Active transport of ions*

Since neither impermeability nor ion binding can be demonstrated to play dominant roles in cells and protoplasm, it is only natural that recent investigations have been interpreted from the point of view of active transport of ions. Such mechanisms can give rise to 'physiological impermeabilities' but still be reversible and dynamic. For our purposes, active transport may be defined as any process leading to the movement, into or out of a fixed volume, of the substance in question against its electrochemical gradient. This is the simplest case to consider, since the complications introduced into systems with bulk movement of fluids are considerable. With specific reference to the ions, sodium and potassium, not only can a constant volume of the muscle fibre usually be assumed but also a constant number of base-binding groups per unit of protoplasmic volume. This assumption, however, should always be regarded with suspicion, since it does not hold for some muscle types nor need it always hold for the same muscle under different conditions (cf. Steinbach, 1947).

The electrochemical gradient for a charged unit such as an ion is represented by the sum of the diffusion gradient and the electrical gradient. The details of operation of these gradients have been worked over admirably by others reporting in this symposium. At electrochemical equilibrium the electrical potential difference between two phases is a function of the ratio of concentrations of any given mobile charged unit in the phases multiplied by the appropriate constant and sign. This, of course, holds only for an equilibrium state. The relationship between potential difference and concentration ratio during attainment of equilibrium is not so simple, nor has it been well worked out in a way useful to the biologist.

As has been known for many years, the potential difference between the inside and the outside of the muscle fibre is very close to that calculated from the ratio of potassium concentrations. Hence muscle fibres (and cells in general) have been referred to frequently as potassium electrodes. In a sense, then, there is nothing to explain about the potassium distribution; it is just as it should be, granted that there is some device for producing the electrical potential difference. With the knowledge that sodium, of similar charge to potassium, can penetrate into and out of muscle fibres and yet be maintained in low internal concentration, distinctly not in electrochemical equilibrium with external solutions, emphasis has shifted to sodium transport systems as devices leading to the potassium equilibrium.

While evidence for the active extrusion of sodium from the interior of frog muscle fibres has been available for some time, recent work has been concerned with two types of phenomena: (1) the demonstration that the exchange of sodium and potassium between inside and outside of the fibre is completely reversible (Steinbach, 1950), and (2) the demonstration with isotopic tracers that influx and efflux of sodium are distinctly different (Levi & Ussing, 1948). In addition, it has recently been shown that no potassium accumulation is noted when internal sodium of sodium-enriched muscles is replaced by choline (Steinbach, 1952). Choline appears not to be involved in the transport mechanisms, therefore it is not extruded and hence there is no space (charges) made available in which potassium can be concentrated rapidly in excess into the interior of the fibre according to the electrochemical gradient. In general, the evidence available at present is entirely consistent with the idea that the major system for regulating the sodium and potassium balance of the frog sartorius fibre is a sodium extrusion system. It should be noted, of course, that a contribution by a potassium transport system is by no means ruled out. It merely is not necessary to explain results at hand.

Assuming the validity of the foregoing analysis, some new evidence will be presented relating to the general characteristics of the sodium transport system. These results, which will be reported in more detail elsewhere, have all been obtained by methods previously described (cf. Steinbach, 1952).

Sodium-rich, potassium-poor sartorius muscles were prepared by soaking pairs of muscles overnight at ice-box temperatures (*c.* 2° C.) in potassium-free Ringer's. Rate of loss of potassium and rate of gain of sodium is by no means constant for different sets of muscles, but the rates are more nearly comparable between members of a pair. Therefore most results are expressed in terms of differences between members of pairs of muscles. In general, one muscle of a pair, after equilibration with the potassium-free salt solution, was analysed as an initial control, the other member of the pair being treated with potassium-containing solution as the experimental muscle. It will be assumed throughout the presentation of the data that sodium extrusion is the primary process. However, it must be remembered that, operationally, rate of sodium extrusion is identical with rate of potassium uptake.

The rate of sodium extrusion

By exposing experimental sodium-rich muscles to potassium Ringer's (0·01 potassium, slightly above the minimum maintenance concentration) for different periods of time the data obtained in Fig. 1 were obtained. It is clear that the major fraction of the excess sodium has been extruded during the first 30 min. of recovery at room temperature. Since about 15 min. is necessary for equilibration with the extracellular spaces (Levi & Ussing, 1948), it is obvious that sodium extrusion is a very rapid process under these conditions. Without attempting any precise corrections for diffusion into extracellular spaces, a rate of 40 mmol./kg./hr. may be set as a reasonable rate.

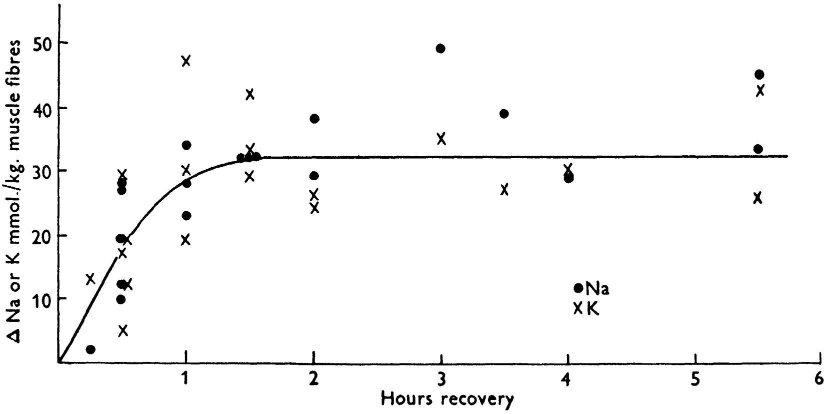

Fig. 1

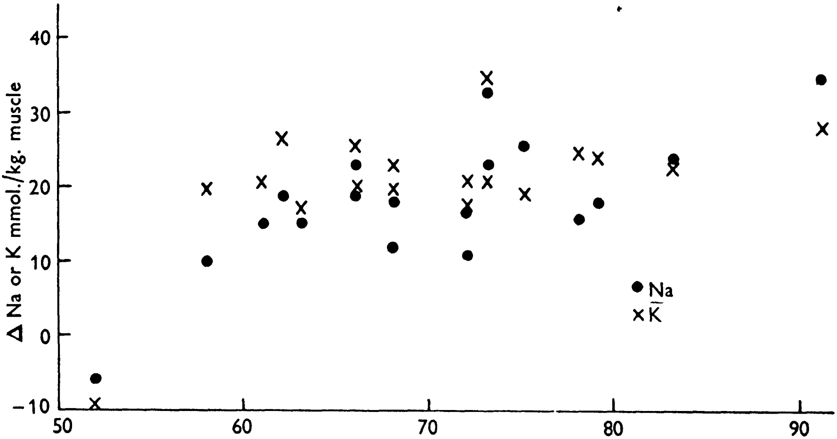

Fig. 2

The effect of internal sodium concentration on the rate of sodium extrusion

Taking advantage of the normal variability of rate of uptake of sodium during the initial equilibration period, the data of Fig. 1 may be replotted to show the relationship between internal sodium concentration and rate of extrusion of sodium. For this plot it is assumed that the sodium extrusion during the first 30 min. of recovery can be used as an index of initial rate. Again, no attempt has been made to make these figures more precise by correcting for extracellular space diffusion.

Fig. 2 shows that, when muscle sodium is above about 60 mmol./kg., the rate of sodium extrusion is independent of the internal concentration. At lower internal sodium concentrations, the initial rate of extrusion is less. This, of course, is the behaviour that would be expected of a saturable carrier mechanism of sodium extrusion.

The work previously cited on substituting choline for sodium in internal and external fluids (Steinbach, 1952) also gives evidence that the rate of sodium extrusion is *independent* of external sodium concentration at all levels from isotonic to one-tenth isotonic.

The effect of external potassium concentration on sodium extrusion

For each sodium ion that is extruded to the outer medium without an anion, some other cation must enter to maintain electro-neutrality. Normally this would appear to be accomplished by entrance of potassium, presumably by diffusion along an electrochemical gradient. If this is true, then the rate of inward diffusion of potassium might well be a limiting factor for outward sodium extrusion. To test this, initial rates of sodium extrusion were measured when sodium-rich muscles were immersed in recovery solutions containing different concentrations of potassium. The results, presented in Fig. 3, show that when external potassium concentration is about 0·02 M the rate of sodium extrusion is optimal. Lower external potassium concentrations result in lowered rates of sodium extrusion.

Variable external potassium concentrations have many different effects on the frog sartorius (Sandow & Kahn, 1952), so the interpretation of these experiments is by no means easy. However, the provisional conclusion may be drawn that inward leakage of potassium may be a limiting factor for outward sodium extrusion.

The temperature coefficient of sodium extrusion

Table 1 summarizes data relating to the effects of temperature on the rate of sodium extrusion. The data were obtained for only two temperatures. A Q_{10} value calculated for the range between these two points is above 3·0.

III. THE MECHANISM OF SODIUM TRANSPORT

The results of experiments outlined above are all consistent with the following conclusions:

(1) Sodium is moved at a rapid rate outward against an electrochemical gradient. Compensation for positive sodium charges moved outward is

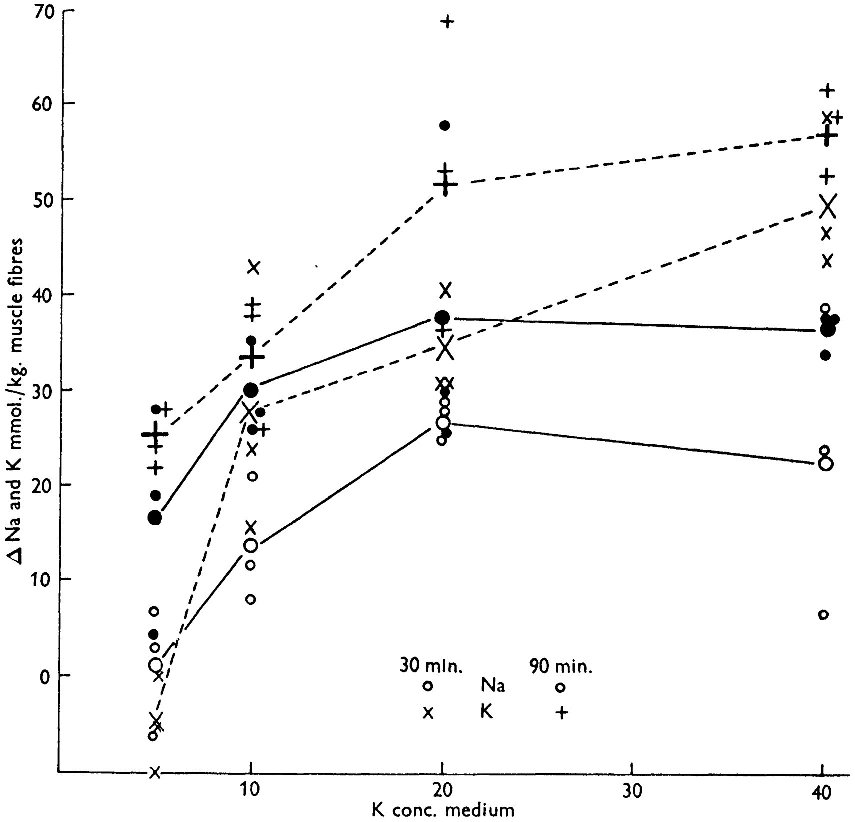

Fig. 3

Table 1. *Average changes in sodium and potassium of muscle fibres during recovery at 4° C. and at 22° C. Eleven pairs of muscles for each temperature, sodium and potassium analyses on each muscle.*

For calculating fibre concentrations as mmol./kg. fibre, an interspace value of 25 % is assumed. Statistical treatment of data according to Simpson & Roe, *Quantitative Zoology*.

	Change in concentration			
	Na		K	
	22° C.	4° C.	22° C.	4° C.
Average change*	−13	−2	15	3
σ'_M	2·3	1·5	3·4	1·4
Difference 22°–4	−11		12	
σ'_d	2·8		3·7	
d/σ'_d	3·9		3·2	
't'	4·15		3·4	

* Average change: concentration in fibres at end of 30 min. recovery minus concentration of control member of pair at end of extraction period and at zero recovery time.

effected by the inward diffusion of potassium along an electrochemical gradient.

(2) The rate of sodium transport outward may be so fast that the rate of inward diffusion of potassium becomes a limiting factor.

(3) With high-sodium concentrations in the fibre substance, rate of extrusion of sodium is independent of internal sodium concentration. With low internal sodium concentrations, extrusion outward is a function of internal concentration.

(4) The rate of outward extrusion of sodium is independent of external sodium concentration.

(5) Sodium extrusion is highly temperature-sensitive with a Q_{10} above 3·0.

All of these conclusions suggest that sodium transport is effected by some specific chemical carrier device, presumably located near the external limiting boundary and present in an effective concentration which can become limiting at high internal sodium concentrations. Furthermore, combination with sodium to give net outward transport appears to take place at the internal surface, not the external. This might merely mean that there are more active combining groups directed inwardly than there are directed outwardly.

Direct chemical evidence on the nature of specific sodium transport mechanisms is not at hand. Various vehicles, such as non-aqueous organic radicals, phosphate combinations and lashing protein tails, have been proposed from time to time. In frog muscle homogenates an excess, amounting to a small percentage of the total sodium, is found to be bound to the sedimentable particulate matter (Steinbach, 1952). At the moment, there is no special reason for implicating any one type of compound. It should be emphasized, however, that the carrier compound need not be concerned exclusively with sodium or any other single ion. All that is required is a discrimination such that the outward movement of ions by the carrier pathways should have a higher sodium/potassium ratio than the inward leakage, presumably by diffusion through pores.

The requirements may be illustrated by a few simple considerations. The electrical resistance of the fibre membrane is known to be high; a figure of 1000 ohms/cm.^{2} may be assumed. If all of the ions carrying current through this resistance are regarded as moving through a water-filled hole, then that hole need occupy an area of the order of only one-millionth of the total surface. Therefore there is a great expanse of surface available for specific chemical transport systems. Calculations show that even assuming improbably slow turn-over values for carrier molecules, maximum rates of transport could be accommodated without crowding of carrier molecules at the surface.

If we assume that the external solution has a sodium/potassium ratio of 10 and that diffusion rates within the membrane are in the ratio of 50 for potassium to 1 for sodium, then, with zero concentration of both ions inside originally, the first ions to penetrate would give a sodium/potassium ratio of 0·2, or an accumulation of potassium. However, as diffusion went on with no increase in volume, the internal ionic ratio would approach that of the external fluid, unless there were some outwardly directed carrier system that had an effective association constant for its sodium compound that was at least five times greater than the association constant for the corresponding potassium compound. Furthermore, some mechanism must exist for the creation (or rejuvenation) of this carrier substance within the fibre and its destruction externally. Our present information does not allow us to attempt to name specific compounds. The presence of esterases on the outside of yeast and other cells might implicate esters as part of the carrier system, and the polyphosphates are known to be effective binders of alkali and alkaline-earth metals.

IV. THE PHYSIOLOGICAL ACTION OF INTRACELLULAR SODIUM AND POTASSIUM OF MUSCLE

There have been many investigations reported in the literature on the effects of alteration of the sodium and potassium concentrations of the environmental fluids on muscle action. In general, two types of effect may be distinguished: the one a rapid effect, occurring soon after application of the agent presumably to be ascribed to alterations of external limiting layers and the other a long-delayed effect, occurring after half an hour or so of treatment and which may be related to intracellular changes in the bulk concentration of the ions of the protoplasm. A recent paper by Sandow (Sandow & Kahn, 1952) reviews some of this literature and presents new information.

Recently we have carried out a few preliminary investigations of tension development by sodium-enriched muscles and muscles after they have recovered for a short period in the high-potassium medium. Isolated sartorii, treated as usual with a preliminary soaking in potassium-free solution, were tested with short tetanic stimuli from an induction coil. Tensions were registered with a simple torsion-wire myograph. Since muscles immersed in the usual high-potassium recovery fluid (containing 0·01 M potassium) are non-irritable, all muscles were tested for tension development only after equilibration for half an hour in the potassium-free medium. Seventeen muscles, soaked 24 hr. in potassium-free solution, gave an average tension of 94 g./g. of muscle. The corresponding control muscles soaked for the same period in high-potassium solution gave an average tension of 256 g./g. of muscle. During this period of soaking other sets of muscles lost approximately one-third of their potassium with a corresponding gain of sodium. If the individual results are plotted there is an indication of an almost linear relationship between the *highest* tension observed at a given internal potassium concentration and the internal concentration of potassium. Marked deviations toward abnormally low tensions are observed, however, leading to the conclusion that tension is related to internal potassium concentration but also to other unknown factors. In another series of twelve pairs of muscles, all muscles were soaked overnight in the potassium-free medium. One member of each pair was then allowed to 'recover' for 1 hr. in the high-potassium medium, then washed in potassium-free medium to develop irritability, and tensions developed were compared with the controls which had been carried through similar manipulations in the potassium-free medium. The 'recovered' muscles gave an average tension of 150 g./g. of muscle, the unrecovered controls, 98 g./g. Again, the variations were great so the results must be regarded as preliminary. However, they are entirely consistent with other observations on whole animals which show that muscles in animals maintained on a low potassium diet, and which develop low-potassium, high-sodium muscles, are weaker muscles than normal controls (Heppel, 1939).

Preliminary measurements of membrane potential have also been made, using the Ling & Gerard (1949) technique of membrane puncture with microelectrodes. We confirmed their results completely with normal muscles in the usual Ringer's fluid (0·0025 M potassium), the average membrane potential being 93 mV. Muscles soaked overnight in the high-potassium fluid (0·01 M potassium) and measured immediately after removal to room temperature in the same medium gave an average potential of 54 mV., decreasing to 50 mV. in half an hour. Similar muscles, soaked overnight in the potassium-free medium and removed to the high-potassium medium and room temperature for measurement gave an average membrane potential of 46 mV. immediately after exposure to the high-potassium and 48 mV. after half an hour recovery. The change during the recovery period is not statistically significant, but the fact that the control muscles showed a consistent *decrease* in membrane potential during the same period probably indicates some significant effect of the extrusion of sodium and uptake of potassium that was occurring in the potassium-depleted tissues. It should be noted, however, that the loss of potassium during the soaking period must have amounted to 30–50% of the initial potassium of the muscle. Hence, if the membrane potential measured really reflected the ratio of potassium, internal to external, it should have decreased by from 40 to 70%. Since the decrease amounted to less than 20% it seems obvious that the measured membrane potential depends upon other factors than the potassium gradient. It should be recalled that Tobias (1950) showed that waterlogged muscles after all the potassium had been removed by distilled water still showed respectable membrane potentials.

V. EFFECTS OF SODIUM AND POTASSIUM ON MUSCLE CONSTITUENTS

While it is true that ionic strength has a marked effect on the activity of the acto-myosin system, no clear-cut distinction can be made between sodium and potassium (cf. Szent Gyorgi, 1951; Mommaerts, 1950). So far as the actual contractile machinery is concerned, there appears to be no property which might be expected to be altered greatly by change of the intracellular concentrations of the two ions. However, if we turn our attention to the enzyme systems, most of which might be expected to be concerned with the flow of energy to the contractile system (recovery or charging-up), there are many examples of specific effects of potassium and sodium. In general, potassium is stimulatory, sodium is inhibitory, although the two need not be antagonistic (cf. Utter, 1951; von Korff, 1953). From the variety of information available about ion effects on enzymes, it is difficult to make much physiological sense. Most ion effects on enzyme systems have been reported on extracts, and it is known that the effects of a given agent on a soluble enzyme may be quite different from its effects on the same enzyme attached to the structural elements of protoplasm (cf. Steinbach, 1949). However, based mostly on studies of soluble systems, some generalizations may be hazarded. In the first place, in those instances where potassium activates an enzyme system (mostly phosphate transfer systems), the activation is maximal over a broad range covering the concentration of potassium normally found in muscle (i.e. near 0·1 M). No sharp 'cut-off' points are reported, although there are a few systems which will not function at all in the complete absence of potassium. If potassium exerts similar effects on enzymes in cells then it seems rather improbable that potassium, as an activating ion, has much of a regulatory function. From all of our available knowledge, it would seem probable that the normal concentration of potassium is always higher than is necessary for very adequate activation of the enzyme systems concerned with glycolysis and phosphate transfer. Therefore potassium within the cell is probably to be regarded as almost indifferent in the sense that there is so much of it around that the interior never need worry about a lack. So far as potassium is concerned, activation is probably always normally maximal.

With sodium, however, in those instances where there is a pronounced sodium inhibition, the case is otherwise. For example, the important acetate-activating system is 50% inhibited by 2×10^{-2} M-sodium (von Korff, 1953). This concentration is seldom found in the interior protoplasm of the frog muscle fibre but might well be present in the outer layers concerned with sodium transport. Any displacement of the sodium from these layers by an indifferent ion such as potassium would then not only release an inhibition rapidly but would also have its effect in a highly reversible fashion due to the interaction of sodium and potassium. Since the acetate activation system is already strongly implicated in surface phenomena concerned with excitation, the pronounced sodium inhibition becomes of considerable interest. The surface of the resting muscle fibre, complete with sodium transport mechanism concentrating sodium from the interior and moving it outward to a strong sodium environment, probably does represent a 'high-sodium' area of the cell, where the acetate-activating enzymes are held inhibited. This inhibition might be presumed to be released immediately following stimulation due to the formation of a new mixing zone in the outer boundary where the sodium concentration would be lower than normal and the potassium concentration would be higher.

In addition to effects of sodium and potassium on enzyme systems, other effects due to structural alterations might be expected. Sodium, a compact ion of high charge density might be expected to promote complex formation much more than potassium which is larger with lower charge density (cf. Steinbach, 1952). Differences of mobility of the two ions have been strongly emphasized in the past. However, so far as the physiological effect is concerned, it would seem probable that the *effect* of an ion depends more upon what it *does* when it gets to its site of activity than upon the *speed* with which it gets there.

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RELATIONS BETWEEN ACTIVE TRANSPORT AND METABOLISM IN SOME ISOLATED TISSUES AND MITOCHONDRIA

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I. INTRODUCTION

Most of the early work on what we now call active transport consisted of investigations with the whole animal and was a study of secretion and absorption. This has given information about the nature of the secretions produced by organs such as the stomach, pancreas and intestines, and about the way in which the activities of these organs are controlled in the whole animal. For more than fifty years it has been possible to study secretory activity in perfused organs, but these approaches are not suitable for finding out what goes on inside the cells of these organs, and this is only possible when investigations are made with much simpler systems under controlled conditions and with quantitative measurements. Within the last few years this has been accomplished and experiments have been carried out in many laboratories on isolated slices or sheets of tissue which have been handled by normal biochemical techniques. These investigations have thrown light on some details of the mechanisms of secretion and accumulation and have made possible comparisons between the processes of metabolism, which supply energy, and of active transport, which require energy supplies. This has led to the discovery that the whole cell is not the simplest unit which is able to maintain active transport. It is now known that the mitochondria, which are the structures responsible for virtually all the respiratory metabolism of cells, are able both to secrete and to accumulate a variety of inorganic and organic cations and anions. This last conclusion comes from a line of work which we have followed in Sheffield for eight years, and has involved investigations on isolated gastric mucosa, on brain and kidney slices and finally on kidney mitochondria.

II. ION MOVEMENTS IN BRAIN SLICES

The problem of hydrochloric acid production has been reviewed recently and will not be discussed directly here, but instead some of the work at Sheffield on the problems of ion transport in brain and kidney will be considered.

Stern, Eggleston, Hems & Krebs (1949) showed that many isolated tissues could accumulate L-glutamic acid during aerobic metabolism and found that brain cortex slices were most effective. Although in the presence of glucose these slices could maintain an internal concentration of at least about 20 times that in the medium when this latter was low (approx. 1 mM), the transport of L-glutamate into the tissue stopped when the difference between the internal and external concentrations became about 20 mM. This applied over the range from approx. 1 to 20 mM-L-glutamate final concentration in the external fluid, and this ability to accumulate glutamate against a concentration gradient was inhibited in the absence of oxygen or the presence of 2:4-dinitrophenol.

Further work led to the discovery by Terner, Eggleston & Krebs (1950) that L-glutamate can play an important role in potassium accumulation in brain cortex slices; slices of guinea-pig and rabbit-brain cortex incubated for 1 hr. in a saline medium lost half their potassium ions, but this could be prevented if both glucose and L-glutamate were added to the medium. Once again this effect could be inhibited by 2:4-dinitrophenol and failed to occur anaerobically.

Ox retina is an easier tissue to handle, and with it they showed that sodium movements were approximately equal and opposite to potassium movements, and that during the recovery phase glutamate and potassium were taken up in approximately equivalent amounts. There is a discrepancy here that has not yet been cleared up. If two positive ions exchange and glutamate enters, then, since glutamate is on balance a negative ion, some other ion as yet unknown must move to balance the electric changes.

The recovery phase was characteristic of ox retina which had been brought to the laboratory in ice-cold saline and was found to have a relatively low potassium and high sodium content.

However, a recovery phase was also found to occur in the brain slices which were found to lose rapidly 40% of their potassium content within a few minutes of the start of incubation at 40° C. and then to re-accumulate this lost potassium during the next half-hour if the conditions were those already described.

This showed the dynamic nature of the processes involved in producing the steady level of potassium in the slices; so the next step was to find out if this steady level of potassium was itself a reflexion of to-and-fro movements of this ion. This was likely because work with intact animals had shown qualitatively that the potassium ions of animal tissues and body fluids continually interchange (Joseph, Cohn & Greenberg, 1939; Hahn, Hevesy & Rebbe, 1939; Fenn, Noonan, Mullins & Haege, 1941–2; Noonan, Fenn & Haege, 1941).

Experiments with ^{42}K showed conclusively that the potassium was rapidly exchanged even though there were no net changes in concentration (Krebs, Eggleston & Terner, 1951). The average turn-over rates for brain were between 3·5 and 4·0 %/min. and for retina 7 and 10 %/min. These rates are very high compared with the turn-over rate of potassium in red blood cells which is 0·03 %/min. (Raker, Taylor, Weller & Hastings, 1950; Sheppard & Martin, 1950; Solomon, 1950). Thus in brain potassium exchanges on average about 120 times, and in retina about 250 times more rapidly than in human red cells.

The experiments were not designed to study the rate of the net accumulation, but for brain this must have had a Q_K (uptake) of at least -13 expressed as µl./mg. dry wt./hr. (where 22·4 µl. = 1 µmole). This can be compared with the steady-state exchange with a Q_K (exchange) of ± 32. The rate of oxygen uptake expressed in the same units was $Q_{O_2} = -18$. On the assumption that this turn-over was caused by an active uptake and a passive leakage Krebs *et al.* (1951) calculated that this turn-over rate would require at least 2·5 % of the energy available from metabolism in brain.

Since it was not easy to test this assumption in brain tissue because of the swelling that occurs during incubation, Mr R. Whittam and I turned to kidney to investigate the problem of the turn-over of sodium with this tissue.

Now work with frog muscle and giant axons of invertebrates, which have a negative intracellular electric potential, had made it probable that a sodium pump operates to maintain the observed ionic concentration differences in these tissues (Dean, 1941; Krogh, 1946; Hodgkin, 1951), and that potassium movements are largely or entirely passive with no change in electrochemical potential. Thus the work done in moving this ion inside the cell against a concentration gradient would equal the work gained by moving the positive charges to the negative inside of the cell (cf. Ussing, 1952).

Although the electric potentials inside brain or kidney cells have never been measured, it was important to investigate sodium movements and turn-over in case a sodium pump operates in these tissues.

The swelling of brain slices in saline solutions, which has been mentioned already, was discovered by Elliott (1946), and Stern *et al.* (1949) showed that the slices swelled about 25 % aerobically but 50 % anaerobically; thus respiration is an important factor in controlling the fluid uptake of the tissues. They also found that kidney slices maintained their original wet weight during incubation, and this makes them suitable for investigations on sodium turn-over which would be difficult with tissues which swell, because of the problem of allowing for the sodium in the fluid imbibed by the swollen tissue.

III. ION MOVEMENTS IN KIDNEY SLICES

Net changes of ions

It was first necessary to find suitable experimental conditions and to measure the sodium, potassium and water content of the guinea-pig kidney cortex slices under a variety of conditions. It was known from the work of Krebs *et al.* (1951) that when no substrate was added about one-third of the tissue potassium was lost on aerobic incubation, and about two-thirds on anaerobic incubation for 30 min. at 40° C. in a bicarbonate saline containing 5 mM-potassium ions and gassed with 5 % carbon dioxide in oxygen. Addition of α-ketoglutarate prevented this loss, L-glutamate and L-aspartate reduced it, but pyruvate, succinate, citrate, fumarate and glucose had no effect.

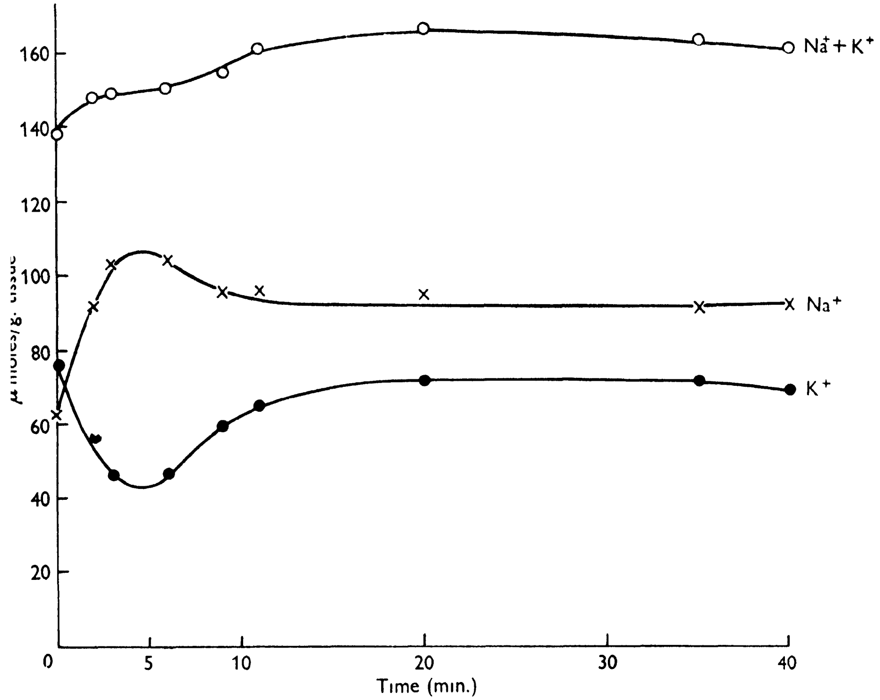

Fig. 1. Changes in the sodium and potassium content of guinea-pig kidney cortex slices on incubation *in vitro* at 37° C. in bicarbonate saline containing 155 mM-Na^+, 5 mM-K^+ and 10 mM-α-ketoglutarate^{--}; gas 5 % CO_2 in 95 % O_2.

The initial experiments showed that kidney cortex behaved in a very similar way to brain cortex. The tissue was sliced with a dry razor and on aerobic incubation at 37° C. it was found that there was an immediate loss of potassium and gain of sodium during the first 2 or 3 min. (Fig. 1).

These changes were largely reversed during the next 20–30 min., after which a steady state was maintained for a further 40 or 50 min. After still longer periods the slices began to leak out potassium once again and the sodium content increased.

Table 1. *The relation between the changes in sodium and potassium and the net accumulation of α-ketoglutarate during aerobic incubation of guinea-pig kidney cortex slices for 40 min. at 37° C. (± standard error)*

All concentrations given as μmoles/g. tissue

		No. of exps.
ΔK^+	$= -\ 1{\cdot}1 \pm 1{\cdot}9$	19
ΔNa^+	$= +26{\cdot}8 \pm 2{\cdot}58$	14
$\therefore\ \Delta\,(Na^+ + K^+)$	$= +25{\cdot}7 \pm 1{\cdot}58$	33
Δ α-ketoglutarate^{--}	$= +12{\cdot}0 \pm 0{\cdot}58$	13
$\therefore\ \dfrac{\Delta\,(Na^+ + K^+)}{\Delta\alpha\text{-ketoglutarate}^{--}}$	$= +\ 2{\cdot}1 \pm 0{\cdot}17$	—
α-ketoglutarate^{--} metabolized in 40 min.	$=\ 53{\cdot}3 \pm 5{\cdot}46$	15

Table 2. *Effect of α-ketoglutarate concentration on the potassium content of guinea-pig kidney cortex slices*

Slices (150 mg.) incubated at 37° C. for 40 min. in 2 ml. bicarbonate saline gassed with 5 % CO_2 in O_2.

Initial α-keto-glutarate concentration in medium (mM)	Amount of potassium in tissue (μmoles/g. tissue)		
	Before	After	Change (%)
0	78·8	63·3	−20
2	78·8	65·8	−16
4	78·8	73·7	− 6·5
6	78·8	78·3	− 0·6
8	78·8	78·5	− 0·4
10	78·8	79·5	+ 0·9

The gains and losses of sodium and potassium were approximately equal and opposite, but, whereas the lost potassium was completely recovered under these conditions, some of the sodium was not extruded. Measurements of the pH of the medium with a glass electrode showed that no changes at all could be detected, so some other ion appeared to be involved since the excess of positive ions accumulated had to be accounted for somehow.

Measurements were therefore made of the movement and metabolism of the substrate α-ketoglutarate with results given in Fig. 1 and Tables 1 and 2. It was first confirmed that concentrations of α-ketoglutarate lower than 6 mM were less effective in maintaining the potassium concentration (see also Krebs *et al.* 1951), and found that there was an accumulation of α-ketoglutarate inside the slices. Although the fresh slices had a very low content of this anion (0·15 mM) this increased during incubation and could reach at least 2·5 times the concentration in the medium. This is comparable with the situation in brain where the specific substrate required for potassium re-accumulation, the L-glutamate anion, is also accumulated against a concentration gradient. If, as is likely in both brain and kidney, the insides of the cells are negative relative to the outsides, then these accumulations of L-glutamate and α-ketoglutarate must be accomplished not only against concentration gradients but against electric potential gradients. This is a process for anions quite similar to the extrusion of the cation, sodium, in nerve and muscles which takes place against an electrochemical gradient.

In these experiments on kidney, in contrast to those on brain, there was no ionic discrepancy, since the increase in sodium plus potassium, which was almost all an increase in sodium, was exactly twice the increase in α-ketoglutarate ions. This means they were electrically equivalent, since α-ketoglutarate is a dibasic acid and virtually completely ionized at the experimental pH values. Another difference between the two tissues is that in brain both glucose and L-glutamate are required to recover the potassium, whilst in kidney α-ketoglutarate is completely effective on its own and the addition of glucose makes no difference.

It is of interest that four times as much α-ketoglutarate is metabolized and disappears from the system as is accumulated and found inside the tissue slices (Table 1). This makes it probable that the actual accumulation is much greater than the net accumulation because all oxidation of α-ketoglutarate occurs inside the cells on the mitochondria. Further work, which is in progress, is therefore needed on the fate of this substrate before the situation can be clarified.

When these preliminary experiments had been completed Mudge (1951*a*) published experiments on rabbit-kidney cortex slices with rather different experimental conditions. The kidney cortex was wet sliced at 2° C. and the slices were then leached in 0·15 M-sodium chloride for 2–3 hr. at room temperature and kept for a further 40 min. at 2° C. in the absence of substrate, phosphate buffer, calcium or potassium. They were then incubated at 25° C. in a medium containing all these substances but with a potassium content of 10 mM, which is twice that in a physiological saline solution.

Under these conditions Mudge (1951*a*) found that after the period of leaching and cooling the slices had lost potassium and gained sodium. He showed that during the subsequent incubation these changes could be largely reversed and stated that 'Changes in tissue Na are the reciprocal of K'. However, his results all showed an overall increase of sodium in the tissue even when the lost potassium had been recovered. This increase was probably associated with an accumulation of the substrate, but this is uncertain because no measurements of substrate changes were recorded.

Mudge (1951*a*) found that several substrates were effective in supporting ion movements, and these included pyruvate, succinate, citrate and fumarate which Krebs *et al.* (1951) had found to be ineffective. The explanation for this is not clear, but it may reside in the use by Mudge (1951*a*) of a relatively low experimental temperature and a high potassium content in the medium, both of which may help to make it easier for the slices to move these ions, and thus allow other substrates, besides α-ketoglutarate, to support the active transport.

This suggestion that the conditions are less critical at 25° C. than at 40° C. is supported by our finding that inhibitors of carbonic anhydrase lower the potassium content of kidney slices at 40° C. (Davies & Galston, 1952) but not at 25° C. (see also Mudge, 1951*b*).

The initial rates of ion movement found by Mudge (1951*a*) (calculated from Figs. 2 and 3) were a Q_K (accumulation) of -12 and a Q_{Na} (extrusion) of $+16$. However, if the rate of sodium extrusion is taken from the tangent of the smooth curve rather than from the first two points, the Q_{Na} becomes $+33$. These results were with 0·01 M-acetate as substrate at 25° C. and the Q_{O_2} was only $-3{\cdot}8$.

The net rates found in our experiments are given in Table 3. It should be noted that the initial rates during the recovery phase are likely to be too low because under our conditions there was a smooth transition from the period of leakage to the period of recovery. The results are therefore minimal values.

Table 3. *Rate of respiration and maximum net rates of change of sodium and potassium in guinea-pig kidney cortex slices*

Incubated aerobically at 37° C. in bicarbonate saline containing 10 mM-α-ketoglutarate cf. Fig. 1). All Q values are in μl./mg. dry wt./hr., where 22·4 μl. = 1 μmole.

Ion change	Q
K^+ leakage out	$+67$
K^+ accumulation	-25
Na^+ leakage in	-93
Na^+ extrusion	$+11$
Oxygen uptake (20–60 min.)	$-23{\cdot}3$

Before considering the dynamic exchanges during the maintenance of the steady-state conditions there are a few other points relevant to the net changes observed. These concern the importance of aerobic metabolism, the effects of temperature and the presence of a water pump.

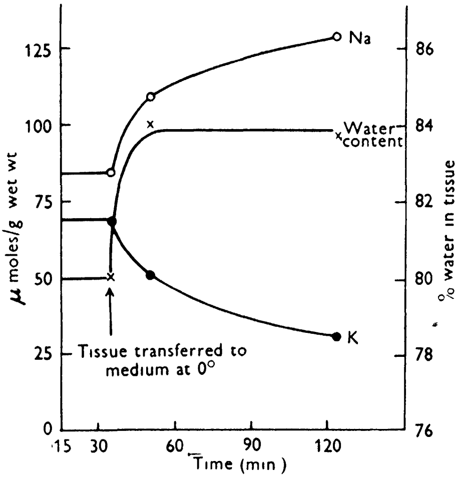

Fig. 2. Effect of 2 × 10^{-4}M-2 : 4-dinitrophenol on the sodium and potassium content of guinea-pig kidney cortex slices incubated aerobically at 37° C. with 10 mM-α-keto-glutarate as substrate.

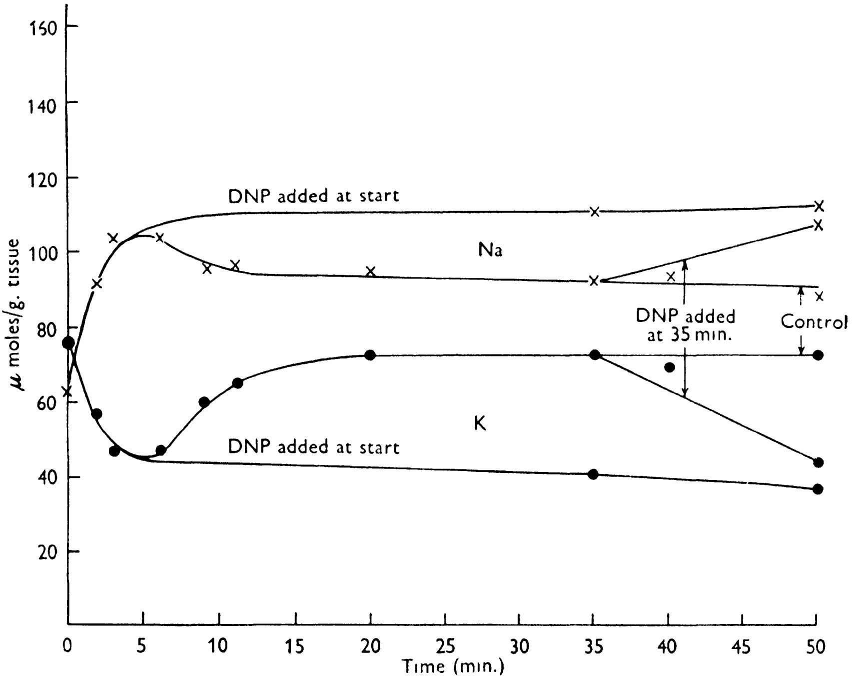

Fig. 3. Changes in sodium, potassium and water content after transferring guinea-pig kidney cortex slices, which had been pre-incubated aerobically at 37° C. for 35 min., to the same medium at 0° C.

The accumulation of potassium and α-ketoglutarate, and the extrusion of sodium all depend on active aerobic metabolism and did not occur either anaerobically at 37 or 0° C. when there is no respiration, or aerobically at 0° C. when respiration is very low. At 0° C. α-ketoglutarate addition had only a slight effect on the Q_{O_2}, and this substrate was not accumulated. This is in contrast to 37° C., where α-ketoglutarate was accumulated and its addition increased the Q_{O_2} by 69%.

These effects of temperature and lack of oxygen, which were accompanied by a swelling of the tissue slices, were reversible (Table 4) (see also Mudge, 1951*a*; Robinson, 1950*a*; Aebi, 1952). As in the case of brain this swelling was far greater than could be accounted for by the difference between the water content of the hydration spheres of the sodium ions, which increased, and that of the potassium ions, which decreased, in the absence of active aerobic metabolism, so some other mechanism must be invoked for this 'water pump'.

Respiration alone is not sufficient to maintain these manifestations of active transport because 2:4-dinitrophenol could stop them (Fig. 2) without affecting the initial rates of leakage or inhibiting the rate of oxygen uptake, which was in fact somewhat increased (Mudge, 1951*b*; Robinson, 1950*b*). These results make it probable that both ion transport and the ability to maintain the normal water content of the kidney slices are mediated through high-energy phosphate compounds.

However, it seems likely that these two mechanisms are not very closely linked together, for the following reasons. When the slices were incubated at 0° C. swelling was complete within 10 min., but the changes in sodium and potassium were still occurring slowly after 2 hr. (Fig. 3). Under these conditions there was normally almost no gradient of sodium plus potassium between the tissue water and the medium (Table 4), but if the slices were transferred to medium oxygenated at 37° C., water was pumped out of the slices and at the same time they developed a gradient of sodium plus potassium. Thus the total content of these ions increased whilst the water content decreased. These results favour the view that kidney slices actively metabolizing in oxygen are not in osmotic equilibrium but maintain hypertonic internal compartments by the expenditure of metabolic energy.

Steady-state turn-over of ions

As in the case of brain it was important to find out whether the steady-state concentration differences of ions were maintained by the cells because they were impermeable to these cations, or whether there were to-and-fro exchanges, and if so what were the relations between these exchanges and the energy supply.

Table 4. *Sodium, potassium, α-ketoglutarate and water content of guinea-pig kidney cortex slices*

Slices (150 mg.) incubated 30–50 min. in 2 ml. bicarbonate saline containing 10 mM-α-ketoglutarate. The term 'concentration gradient' is the ratio concentration in tissue water/concentration in medium. Results ± standard error. Number of measurements in brackets.

		Concentration of ions in tissue (μmoles/g.)				Water content (%)	Concentration gradients	
		Na^+	K^+	$Na^+ + K^+$	α-Keto-glutarate^{--}		$Na^+ + K^+$	α-Keto-glutarate^{--}
Slices before incubation		63·4 ± 0·9 (14)	76·4 ± 0·9 (19)	139·8 ± 2·1 (14)	0·12 (3)	77·7 ± 0·19 (19)	—	—
Incubated slices								
Temp. (° C.)	Experimental conditions							
37	Aerobic	90·2 ± 1·8 (24)	75·3 ± 1·6 (24)	165·5 ± 2·4 (24)	12·0 ± 0·58 (13)	79·7 ± 0·3 (24)	1·29 ± 0·03	2·5
0	Aerobic	112 ± 2·9 (13)	30·9 ± 0·7 (13)	142·9 ± 3·0 (13)	2·3 (3)	83·0 ± 0·5 (7)	1·07 ± 0·04	0·3
37	Anaerobic	114 ± 2·2 (9)	27·5 ± 1·0 (9)	141·5 ± 2·5 (9)	3·1 (3)	82·7 ± 0·3 (4)	1·06 ± 0·03	0·4
0	Anaerobic	109 ± 2·9 (7)	32·2 ± 1·7 (7)	141·2 ± 3·3 (7)	2·1 (2)	83·5 (2)	1·05 ± 0·04	0·3

It was first necessary to find out if all the sodium and potassium in the kidney slices were exchangeable with sodium and potassium in the medium, so slices were incubated in physiological saline solutions containing spectroscopically pure $^{24}Na^+$ or $^{42}K^+$ and were removed after various times. The results are shown in Figs. 4 and 5. They show that, within the experimental errors of 2 or 3 %, the whole of these ions exchanged rapidly, with sodium exchanging faster than potassium. The rates were slower under strictly anaerobic conditions, but the turn-overs were still complete. This appeared to be in contrast to the findings of Mudge (1952), that at 25° C. under anaerobic conditions only 60 % of the potassium in rabbit kidney cortex slices had exchanged after 4 hr. Experiments were therefore carried out at 0° C. with guinea-pig kidney cortex slices and these showed that even after 10 hr. about 20 %, aerobically, and 40 %, anaerobically, of the tissue potassium had failed to exchange despite rapid initial rates of exchange. Thus at 0° C., in addition to the small amounts of extracellular potassium, there were two forms of tissue potassium which exchanged at fast and slow rates (Table 5).

The turn-over rates of both sodium and potassium given in Table 5 were calculated from the experimentally determined curves showing the uptake of the radioactive isotopes with time. In these experiments the slices had been pre-incubated and were in steady states with only slight changes in the concentrations of these two ions. In contrast to potassium the rapidly exchanging extracellular sodium could not be neglected, and the results given refer to the intracellular sodium. Since the concentration of sodium and potassium varied so much with the conditions (see Table 4), the rates per gram of tissue may not be a fair basis for comparison. The rates are therefore given as μmoles turned over per 100 μmoles of intracellular cation in the tissue.

The turn-over rates given in Table 5 for the aerobic slices are the fastest so far found for sodium and potassium in any mammalian tissue (cf. Davies & Galston, 1951), and it is remarkable that in kidney an amount equal to the whole intracellular potassium exchanges in less than 7 min., i.e. more than 200 times a day. Table 5 also shows that, although the contents of sodium and potassium depended on respiration (Table 4), the turn-overs of these ions are largely independent of respiration, the rates in anaerobic conditions being about 75 % of those in the presence of oxygen. The remaining quarter of each turn-over rate is associated with aerobic metabolism, and for sodium, and the slow fraction of the potassium, shows large changes with temperature. The direct effect of temperature on the slowly exchanging part of the tissue potassium was very large, the increase aerobically being 104-fold and anaerobically 184-fold.

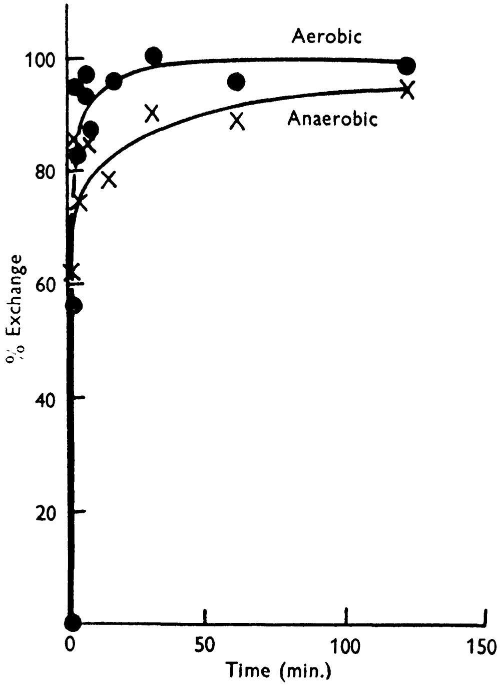

Fig. 4. Exchange of $^{24}Na^+$ in isolated guinea-pig kidney cortex slices at 37° C. Changes in sodium content were occurring throughout the experiment, but from 30 to 50 min. the aerobic slices contained 112 mmoles Na^+/l. tissue water, and the anaerobic slices contained 135 mmoles Na^+/l. tissue water.

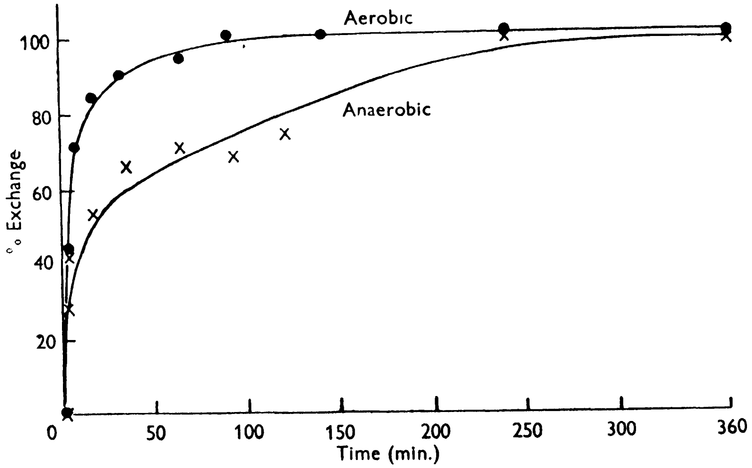

Fig. 5. Exchange of $^{42}K^+$ in isolated guinea-pig kidney cortex slices at 37° C. Changes in potassium content were occurring throughout the experiment, but from 30 to 50 min. the aerobic slices contained 96 mmoles K^+/l. tissue water, and the anaerobic slices contained 35 mmoles K^+/l. tissue water.

These results show the relations between the aerobic metabolism of isolated guinea-pig kidney cortex slices, the amount of water in the tissue and the content and turn-over of both sodium and potassium. It follows that even so-called resting cells must do work continuously to maintain their internal ionic environments. However, the amount of this work cannot be calculated straightforwardly from measurements of the turn-over rates of the ions in aerobic conditions without reference to the situation in, for example, anaerobic conditions (cf. Krebs *et al.* 1951). It thus becomes important to know the location in the cells of the enzyme systems which do the work which maintains this active transport.

Table 5. *Steady-state turn-over rates of sodium and potassium in guinea-pig kidney cortex slices*

(All turn-over rates for sodium refer to the slowly exchanging fraction.)

Ion	Gas phase	Temp. (° C.)	Turn-over rate (μmoles of cation/min./100 μmoles)	Ratio of turn-over rates 37° C./0° C.	Increase of turn-over rate due to O_2 at 37° C. divided by the increase of turn-over rate due to O_2 at 0° C.
Na^+	O_2	37	16·2	1·81	> 20*
		0	8·9		
	N_2	37	12·1	1·35	
		0	8·9		
K^+	O_2	37	15·9	0·88	0·82
		0	Fast 18·1		
			Slow 0·155	104	
	N_2	37	11·5	0·91	—
		0	Fast 12·7		
			Slow 0·0622	184	47
	Zeo-Karb 225 (10–30 mesh) K salt in 0·01 M-KCl	37	11·0	1·6	—
		0	7·1		

* Calculated after making allowance for the maximum possible error in the measurement of the turn-over rates.

IV. ACTIVE TRANSPORT IN MITOCHONDRIA

The experiments just described refer to the activity of intact cells in kidney cortex slices and were done in collaboration with Dr A. W. Galston and Mr R. Whittam. The work to be presented now shows that these phenomena are largely reflexions of more fundamental events at the sub-cellular level, and was done in collaboration with Mr W. Bartley. We now know that for some activities the elementary secretory units are not the cells but the mitochondria.

During the last few years much work has been published on the chemical activities of various fractions of the cell, and this has shown that the mitochondria are responsible for virtually all the respiration and oxidative phosphorylation that goes on in the cell (see reviews by Green, 1951; Schneider, 1953). In view of the connexion between active transport and oxidative phosphorylation it therefore seemed reasonable to suppose that mitochondria were, energetically, closely associated with secretory activity in the cell. Active transport by cells is nearly always in one particular direction, and this means that cells must have special structures which cause the cells to direct secretions to one particular wall, for example, of the cell. In secreting kidney cells the mitochondria are arranged longitudinally from the cell wall bordering the lumen of the tubules, so it seemed worth while investigating the possibility of a still closer association and hence to look for secretory activity in isolated actively metabolizing mitochondria. This possibility was supported by the well-known fact that the osmotic strength and composition of the suspending medium is of critical importance for maintenance of the size, shape and activity of isolated mitochondria. It was also known that potassium was necessary for maximum oxygen uptake in liver mitochondria (Pressman & Lardy, 1952).

Harman (1950) had investigated the distribution of potassium between mitochondria and the suspending solution but did not find any evidence for an accumulation. However, the particles he had isolated had been kept for more than 20 min. at 0° C., and this would have drastically reduced their rate of metabolism and hence any active transport that depended upon a continuous supply of energy. This was confirmed and the technique developed of separating the actively metabolizing mitochondria from the incubation medium as quickly as possible at 20° C. in the high-speed head of an International Centrifuge which took only 24 sec. to pack down the mitochondria in potassium chloride solutions. Under these conditions it was possible to observe a rather labile ability of the particles to maintain concentration gradients which was dependent on the experimental conditions. Table 6 shows that well-oxygenated, actively metabolizing kidney cortex mitochondria can actively transport a wide variety of organic and inorganic cations and anions (Bartley & Davies, 1952). In all the cases shown there were no, or only small, gradients in the initial material, and the extra ions were accumulated during metabolism. At very low external concentrations of sodium (6×10^{-4} M) the freshly prepared material could maintain a ratio of 26. This may have been due to some 'bound' sodium in the mitochondria. Similarly, ratios for magnesium of up to 4·5 were observed, but there were no clear effects of metabolism on the concentration gradients or the absolute content of this ion. It is possible that the gradients for magnesium are maintained by a type of 'binding' process similar to that described recently for calcium by Slater & Cleland (1953). The results given in Table 6 are for the centrifuged material without allowance for the extraparticulate fluid, so the ratios for the cations must be minimum values. The results for the substrate anions are also uncorrected for the amounts used up during the isolation of the particles. This explains the low ratio found for oxaloacetate, and shows that pyruvate, fumarate and α-ketoglutarate were concentrated in the mitochondria. Since both cations and anions can be accumulated and this accumulation depends on metabolism, any explanation along the lines of a Donnan equilibrium seems most unlikely. It is remarkable that an important function of the cell, the ability to pump water, can also be carried out by mitochondria, since the water content of these particles increased in the absence of metabolism and decreased during active metabolism. Similar results with liver mitochondria have been found by Macfarlane & Spencer (1953).

Table 6. *Ratios of internal/external concentrations found for metabolizing skeep kidney cortex in mitochondria at* 20° C.

Substance	Ratio	Concentration in medium after separation (M)
'H^+'	2·5	$1{\cdot}6 \times 10^{-7}$
Na^+	1·5	$2{\cdot}6 \times 10^{-2}$
K^+	2·4	$9{\cdot}0 \times 10^{-2}$
Orthophosphate	6·0	$1{\cdot}9 \times 10^{-4}$
Adenosine polyphosphates	0·7	$4{\cdot}3 \times 10^{-4}$
Pyruvate	1·1	$3{\cdot}5 \times 10^{-3}$
Fumarate	8·0	$2{\cdot}8 \times 10^{-4}$
Oxaloacetate	0·1	$1{\cdot}8 \times 10^{-3}$
α-Ketoglutarate	1·0	$6{\cdot}3 \times 10^{-2}$
Citrate	0·8	$1{\cdot}4 \times 10^{-2}$

Water content of metabolizing mitochondria = 80 %.
Water content of non-metabolizing mitochondria = 91 %.

It was important to find out whether these ionic differences were due to static accretions of bound ions or whether they were manifestations of dynamic activities, and if so what were the relations between the rates of ion exchange and metabolism. Once again this problem could be investigated with the help of radioactive isotopes.

The whole of the sodium and potassium was exchangeable with extremely high turn-over rates. These rates were so high as to be immeasurable with our techniques at 20° C., even in the absence of substrate. Fig. 6 gives the results obtained at 20 and at 0° C. with sodium and potassium. This shows the effect of added substrate at 0° C. on the sodium turn-over. It was found that the increase in Q_{O_2} following the addition of fumarate was only 0·5, but the increase in Q_{Na} in the first minute was 2000. Thus the uptake of one molecule of oxygen was causing the uptake and output of 4000 sodium ions; with α-ketoglutarate this figure was at least 6400 sodium ions. These results appear to rule out many simple ideas on the mechanism of sodium transport. It is possible that the extra oxygen uptake in the presence of substrate is used by the mitochondria to make available carrier molecules which can themselves transport the ions to and fro at very great rates. It is noteworthy that at 0° C. in the absence of substrate the sodium does not exchange uniformly (Fig. 6). There is a fraction which turns over very slowly, but its turn-over rate can be greatly increased by increasing the rate of metabolism of the mitochondria. Thus sodium turn-over is closely linked to mitochondrial metabolism.

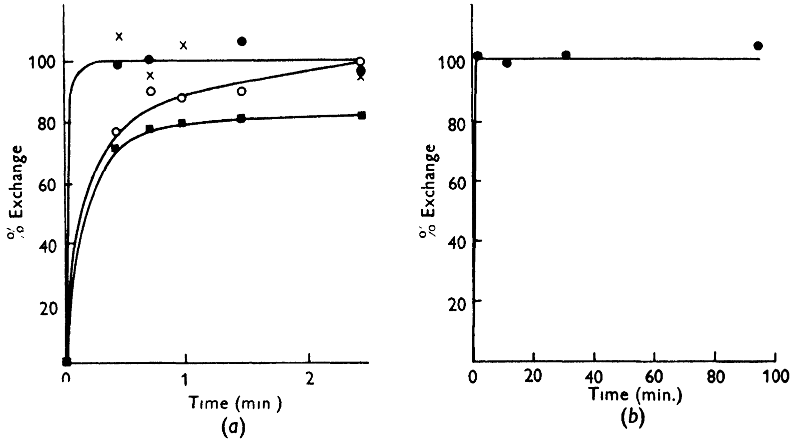

Fig. 6. Exchange of (a) $^{24}Na^+$ and (b) $^{42}K^+$ in sheep kidney cortex mitochondria. (a) × with no substrate at 20° C. (100 % exchange by the first measurement also occurred with 2·5 mM-α-ketoglutarate or 2·5 mM-fumarate at 20° C.); ● with 2·5 mM-α-ketoglutarate at 0° C.; ○ with 2·5 mN-fumarate at 0° C.; ■ with no substrate at 0° C. (b) ● with no substrate at 0° C. (100 % exchange by the first measurement also occurred with no substrate at 20° C. and with 12·5-mM-α-ketoglutarate at 0 or 20° C.).

In the case of potassium the turn-over rate was immeasurably fast under all conditions. It was not possible to show any effect of an increase of metabolism on the rate of potassium exchange, and this means that if the exchange did depend on metabolism then at least 10,000 potassium ions were exchanged for every oxygen molecule used by the mitochondria.

These steady-state exchange rates are so high that they rule out any explanation based on an active uptake and a passive leakage. Two reasons will clarify this. One is that no simple reaction could form a stoichiochemical link between several thousand sodium ions and one oxygen molecule. The other is a difficulty concerning the energetics. Even on the basis of the formation of three high-energy phosphate bonds for each oxygen atom this would only make available 15 cal./mole of sodium ions transported, and this could only produce a concentration gradient of 1·03 even if it were used with 100% efficiency. Much greater concentration gradients are actually maintained (Bartley & Davies, 1952; Macfarlane & Spencer, 1954), so this possibility is excluded.

This means that in these rapid steady-state exchanges there must be energetic coupling of the accumulation and the leakage, and this could occur in two types of way. One way is a balance between two forms of energy, so that, say, a gain of concentration energy goes with a loss of electrical energy as in the exchanges taking place at equilibrium between ion-exchange resins and salt solutions. This is only possible when net transport involves no change in electrochemical potential, and this is unlikely to be the case in mitochondria even if they behaved like a mixture of a cation and an anion exchange resin which could accumulate and exchange both cations and anions. The second way is possible when net transport involves changes in electrochemical potential and must occur in mitochondria. This is the 'exchange diffusion', postulated by Ussing (1949), which requires a fully saturated ion-carrier moving to and fro by thermal motion between the two compartments. Thus when one ion loses energy, another gains it. This allows an exchange of ions to proceed without net transport or an energy supply.

This idea is easier to visualize if one thinks of the Eiffel Tower during peak periods. The top lifts are always full and are linked, so that when one moves up another moves down to balance it. If the lifts were frictionless there would be no net work done. There is, in fact, no net transport of people, but, nevertheless, there is mixing and exchange of those at the top and those at the bottom, and any one individual has had his potential energy increased and again decreased.

The possibility of 'exchange diffusion' must always be borne in mind in interpreting steady-state exchange rates measured with radioactive isotopes. The net uptakes of cations and anions against electrochemical gradients must of course require energy, so an investigation was made into phosphate metabolism which is a link between the energy and the transport.

The results so far obtained throw light on the mechanism by which phosphate is transported by mitochondria and were obtained using radioactive phosphate and radioactive adenosine triphosphate (ATP). In the first experiment a washed sheep kidney mitochondrial suspension in potassium chloride was added to a medium containing pyruvate, bicarbonate, magnesium, chloride, ATP and phosphate buffer, containing labelled phosphate. This system was able to maintain its organic phosphate content and rapidly incorporate radioactivity into the ATP, so that isotopic equilibrium with the phosphate in the medium was 50 %, reached in about 3–4 min. when oxygenated at 20° C. Fig. 7 shows the results obtained when the mitochondria and medium were separated at different intervals of time. In this experiment there was a large accumulation of phosphate within the mitochondria which reached a maximum after 4 min. However,

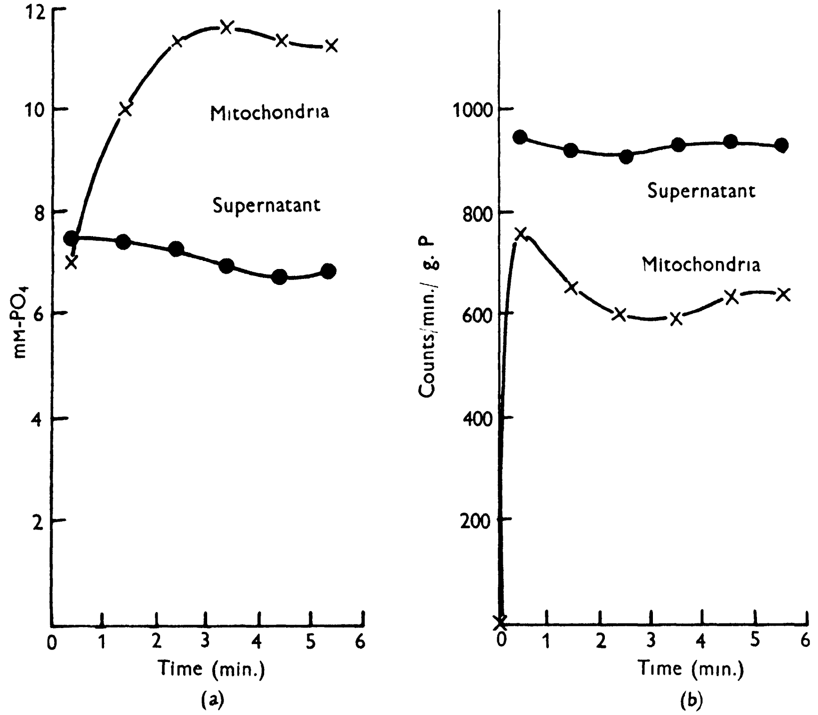

Fig. 7. Changes in the concentration (*a*) and specific activity (*b*) of the orthophosphate in sheep kidney cortex mitochondria and in the medium during incubation at 20° C. with $^{32}PO_4$ in the medium at the start. For further details see text.

the fall in the specific activity of the phosphate in the mitochondrial fraction after the first half-minute in the face of a net increase in the amount of phosphate within the mitochondria and a constant specific activity outside showed that the phosphate accumulating within the mitochondria did not come directly from the external orthophosphate. The only possible source of this non-radioactive phosphate was the ATP in the medium which, on breaking down, was acting as a carrier of phosphate from medium to mitochondria.

It is important to realize that the specific activity calculated for the mitochondrial phosphate includes the activity of the 'extramitochondrial' phosphate in the spaces between the particles. The inulin space of the packed-down suspension was measured and gave the high value of 60%, so these particles may have a system of intercommunicating passages rather like a sponge or loofah. When allowance was made for this, the specific activity of the intramitochondrial phosphate was calculated to be only 50% of the activity of the medium at the first measurement, so some external phosphate must have got in on its own. It is clear, however, from these results that the large increase of non-radioactive intramitochondrial phosphate can only have originated from the added ATP.

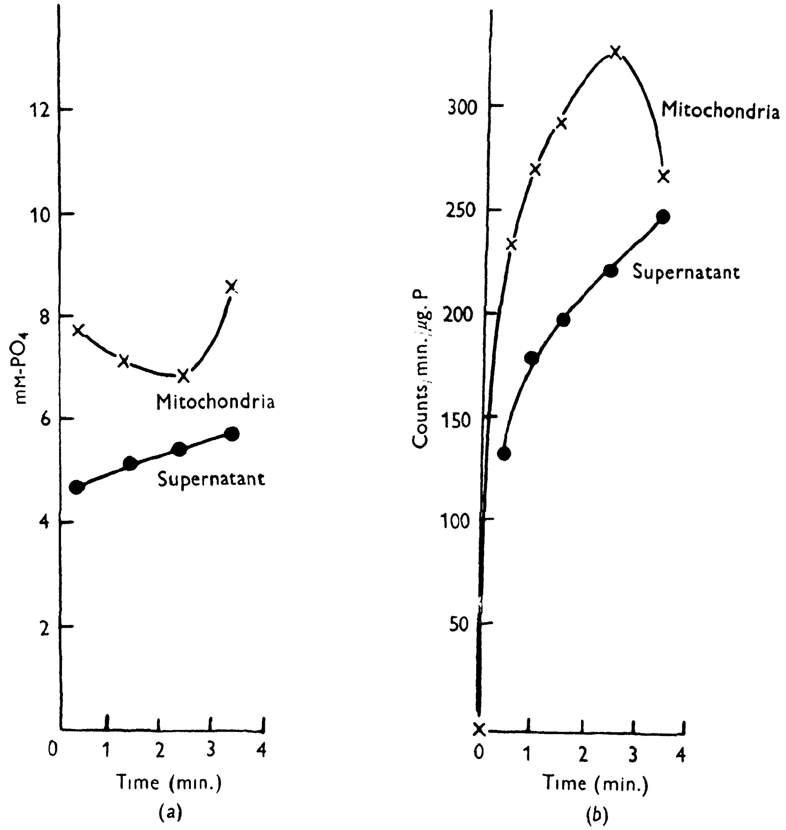

Fig. 8 Changes in the concentration (*a*) and specific activity (*b*) of the orthophosphate in sheep kidney cortex mitochondria and in the medium during incubation at 20° C. with [$\beta\,\gamma-{}^{32}P_2$] ATP in the medium at the start. For further details see text.

This conclusion was confirmed by repeating the experiment with ATP labelled in the two terminal phosphate groups (Fig. 8). These results show that in contrast to the previous experiment the specific activity of the mitochondrial phosphate was higher and increased more rapidly than that in the medium. This could only occur if the mitochondrial phosphate came from the radioactive ATP in the medium. Thus ATP is identified as the carrier molecule for accumulating phosphate from medium to mitochondria (Bartley & Davies, 1954).

These observations with mitochondria are in accordance with similar results for whole cells, since it is now known that the absorption of inorganic phosphate is often a process requiring phosphorylation to form an organic phosphate, rather than a simple exchange of ions across cell membranes. This has been shown for heart muscle by Sacks (1948), for body cells in general by Popják (1950), for red cells by Gourley (1952) and for yeast cells by Nickerson & Mullins (1948) and Spiegelman, Kamen & Sussman (1948). It now seems probable that all these cases reflect the powers of active transport possessed by subcellular particles, and it is predictable that many other types of active transport shown by intact cells will be explicable in terms of the activity of enzyme assemblages within the cells, and in particular of those complex assemblages of enzymes the mitochondria. The multicompartmental structure of these particles has already been demonstrated convincingly by Sjöstrand (1953), and this gives a physical basis for their secretory activity which has to be postulated to explain these experimental results.

V. THE PROBLEM OF THE EFFICIENCY OF ACTIVE TRANSPORT

There have been so many theories put forward recently to account for active transport that there is an urgent need for experiments to test them, rather than for more theorizing. As a contribution to this, Table 7 gives the collected results of the experiments which have been discussed here for the relations between metabolism, net transport and turn-over in those cases where the information is available. These results show that, though the rates of net movement of sodium and potassium against concentration gradients can be accounted for by the possibilities given in Table 7, (*c*) or (*d*), but in many cases not by (*a*) or (*b*), the steady-state rates of ion turn-over must be caused by other mechanisms. This is confirmed by the occurrence of large rates of ion turn-over in anaerobic conditions where net transport against gradients does not occur. Many more such comparisons are required, and a start has already been made for the problem of hydrochloric acid and electricity production in gastric mucosa (Crane, Davies & Longmuir, 1948; Gray, Hokin & Rehm, 1948; Teorell, 1949; Crane & Davies, 1951; Rehm, 1950; Davenport, 1952; Davenport & Chavré, 1951, 1952), of salt uptake in plants (Robertson & Wilkins, 1948*a*, *b*; Lundegårdh, 1949) and of electricity production in frog skin (Francis, 1933; Stapp, 1941; Lund & Stapp, 1947).

Table 7. *The relations between the rates of net movement and turn-over of ions, and the rates of respiration in brain and kidney cortex slices and in kidney cortex mitochondria*

Note. If the ion movements depend on (*a*) organic acids such as lactic or pyruvic produced from glucose during the normal course of oxidative metabolism, then $Q_{\text{ion transport}}/Q_{O_2} \ngtr 0{\cdot}33$; (*b*) the carbon dioxide or carbonic acid produced during the normal course of glucose oxidation, then $Q_{\text{ion transport}}/Q_{O_2} \ngtr 1{\cdot}0$; (*c*) the electrons passing along the cytochrome system, then $Q_{\text{ion transport}}/Q_{O} \ngtr 4{\cdot}0$; (*d*) the number of 'high-energy' phosphate bonds formed, then with a P/O ratio of 3 and 1 ion moved per ~P, $Q_{\text{ion transport}}/Q_{O_2} \ngtr 6$, with 2 ions moved per ~P, $Q_{\text{ion transport}}/Q_{O_2} \ngtr 12$. Ion movements dependent on 'free' or 'exchange' diffusion may be largely or entirely independent of the rate of respiration.

$Q_{\text{ion uptake}}{}^{-}$, $Q_{\text{ion output}}{}^{+}$, $Q_{\text{ion turn-over}}{}^{\pm}$.

Reference	Material	Substrate	Temp. (° C.)	Overall rate of ion movement, $Q_{\text{transport}}$ (μl./mg. dry wt./hr.)	Overall rate of ion movement in absence of O_2, $Q_{\text{transport}}$ (μl./mg. dry wt./hr.)	Rate of respiration Q_{O_2} (μl./mg. dry wt./hr.)	Overall ratio $\frac{Q_{\text{ion transport}}}{Q_{O_2}}$
Krebs *et al.* (1951)	Guinea-pig brain slices	L-Glutamate and glucose	40	− 13 (K^+)	0	− 18	0·7
				± 32 (K^+)	?		1·8
Mudge (1952*a*)	Rabbit kidney cortex slices	Acetate	20	+ 16 (Na^+)	—	− 3·8	4·2
				− 12 (K^+)	—		3·1
Whittam & Davies (1953)	Guinea-pig cortex slices	α-Keto-glutarate	37	− 93 (Na^+)	− 93	− 23·3	4·0
				+ 11 (Na^+)	0		0·47
				+ 67 (K^+)	+ 67		2·9
				− 25 (K^+)	0		1·1
				± 26 (Na^+)	± 49		1·1
				± 80 (K^+)	± 21		3·4
			0	± 53 (Na^+)	± 53	− 0·68	78
				Fast ± 16 (K^+)	± 10		24
				Slow ± 0·2 (K^+)	± 0·07		0·3
Macfarlane & Spencer (1953)	Rat-liver mitochondria	L-Glutamate	28	− 5·8 (Na^+)	− 5·8	− 28	0·21
				− 9·9 (K^+)	− 9·9		0·35
Bartley & Davies (1954)	Sheep-kidney mitochondria	None	0	± 1,600 (Na^+)	—	− 0·5	3,200
				> ± 5,500 (K^+)	—		> 11,000
		Fumarate	0	± 3,600 (Na^+)	—	− 1·0	3,600
		α-Keto-glutarate	0	> ± 4,800 (Na^+)	—	− 1·0	> 4,800
				> ± 5,500 (K^+)	—		> 5,500
Whittam & Davies (1954)	Zeo-Karb 225, 10–30 mesh	None	0	± 350 (K^+)	± 350	0	∞
			37	± 544 (K^+)	± 544	0	∞

There has been much other work on these and other topics, but usually there is some gap in the data so that quantitative comparisons cannot be made. I wish to conclude by appealing to all workers in this field to try and obtain results in two forms if possible, the stoichiochemical and energetic, i.e. the number of ions moved actively in relation to the number of molecules of oxygen used or substrate metabolized, and also, if this can be estimated, the amount of energy needed to drive the active transport relative to the amount of energy made available from metabolism. When sufficient information of this type has become available it should be possible to clear the field of useless theories and make a major step forward in the elucidation of the details of the mechanisms of ion transport.

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ACTIVE TRANSPORT THROUGH EMBRYONIC MEMBRANES

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I. INTRODUCTION

Young birds and mammals acquire passive immunity from their mothers. At the time of hatching or birth they become exposed to the risks of infection, but it is not for some weeks, or even months, later that they can be shown to have developed the power of producing circulating antibodies to antigens which they have themselves encountered. During this interval maternal antibodies are present in the circulation and are partly, if not wholly, responsible for the young animal's powers of resistance to infection. This passive immunity of maternal origin gradually wanes as the young animal develops its own active immunity. At first the titres of antibody of the blood of the young animal may be as high as, or even higher than, those of the blood of the mother.

It is evident, therefore, that the process of transmission of passive immunity from mother to young must be efficient. However, it is known to be accomplished in very different ways in different species. In the fowl the maternal antibodies are secreted in the yolk of the egg while still in the ovary and subsequently are gradually absorbed by the yolk-sac of the developing chick. In the horse, cow, sheep, goat and pig they are present at high titres in the colostrum, or first milk, and at very much lower titres in the subsequent milk. The young of these species are devoid of antibodies at birth, or virtually so, but absorb them through the gut wall with great rapidity from the first feed. Thereafter, the capacity to absorb antibodies from the gut contents declines rapidly and virtually disappears by 24 hr. after birth. In the dog it is probable that some immunity is acquired from the colostrum and milk after birth, the puppy being able to absorb antibodies from the gut contents until 10–12 days old. In rats and mice it seems certain that some immunity can be acquired before birth but that the greater part is acquired after birth, the young being able to absorb antibodies from the gut contents until they are weaned. In man at least the major part of the transference of passive immunity takes place in the uterus but there is weighty evidence of some additional transference by way of the mammary secretions after birth. In rabbits and guinea-pigs transmission takes place before birth, and there is no conclusive evidence of any transfer thereafter by way of the colostrum and the milk. In the rabbit the maternal antibodies are secreted into the uterine lumen during pregnancy and are absorbed by the foetal yolk-sac splanchnopleur. The allanto-chorionic placenta does not appear to play any part in the transmission. Since we have reviewed recently (Brambell, Hemmings & Henderson, 1952) the literature and the experimental evidence for this route of transmission in rabbits we do not propose to dwell on it further here, but to accept this conclusion as established. Unfortunately, at present our knowledge is confined almost entirely to these few species, amongst which there is much diversity.

It is our purpose in this paper to discuss the problem of active transport of proteins in the light of our work on the transference of passive immunity in rabbits.

II. ANTIBODIES AS MARKERS OF SERUM GLOBULIN

Antibodies occur in the globulin fraction of the serum proteins, principally in the γ-globulin. The molecules of γ-globulin which possess antibody activity are specifically adapted to unite with the antigen which has stimulated their production. It is believed that this activity is due to a surface configuration of some of the globulin molecules which renders one or more regions on the surface of each molecule capable of forming a bond with the prosthetic group of the antigen. Apart from this capacity to react with the specific antigen, the antibody is chemically and physically indistinguishable from other globulin molecules of the same serum fraction, and presumably performs the same functions.

Hence, if an immune serum is injected into a non-immune animal, the antibody can be used as a marker of the injected globulin. Appropriate immunological reactions, *in vivo* or *in vitro*, provide tests for the presence of antibodies at very high dilutions; those for antitoxins being particularly useful because they are not only amongst the most sensitive but they are also quantitative. Since antibody activity depends on surface configuration of the molecules it provides a safe means of recognition; any considerable metabolic alteration of the molecule is liable to destroy its immunological activity. The technique is limited in that, *ipso facto*, antibodies can be used as markers of that particular fraction alone of the serum proteins in which they occur, usually the γ-globulin. Immune sera prepared in other species have the advantage that while the antibodies contained in them can be titrated directly, the serum proteins, themselves species-specific antigens, can be recognized as such by precipitin reactions with their homologous antisera. This furnishes a complementary means of establishing the integrity of the foreign protein at least as delicate as that provided by the titration of the contained antibody. Precipitin ring tests are highly sensitive but recognize a serum fraction which is not necessarily coextensive with the antibody. Hence the two tests cannot readily be related quantitatively but have been used to reinforce each other.

Our investigations on the passage of proteins into the foetus have been confined to rabbits. We used antibodies as markers, hence the information obtained was limited to the globulin fraction. The animals employed were not immune, and hence did not make the antibody themselves.

III. THE ARRANGEMENT OF THE FOETAL MEMBRANES

The experiments, the results of which concern us here, were performed on rabbits 24 days pregnant, the immune serum employed being injected into the uterine lumen. The animals were killed 24 hr. after injection, and samples of the foetal fluids and of the maternal blood were then collected and tested for their antibody content. The technique has been described elsewhere (Brambell, Hemmings, Henderson, Parry & Rowlands, 1949) and further detail is unnecessary here.

The arrangement of the embryonic membranes of the rabbit is shown in Fig. 1. It can be seen that the yolk-sac splanchnopleur and the chorion are exposed to the contents of the uterine lumen. The yolk-sac is open after mid-pregnancy, the lower or bilaminar hemisphere having disappeared, and the upper or splanchnic hemisphere being inverted, with its endodermal surface exposed to the lumen but not attached to the uterine wall. This membrane is highly vascular, containing the network of blood-vessels and blood-islands, bounded by the sinus terminalis, which constitutes the area vasculosa. These vessels are connected with the umbilicus by the vitelline arteries and veins in the yolk-sac stalk. The mesenchyme containing the vessels is covered on the side next the uterine lumen by a thick columnar epithelium of endoderm cells resting on a fine basement membrane. The surface of these endoderm cells exposed to the uterine lumen is provided with a typical brush border (Morris, 1950). On the side away from the uterine lumen, the vascular mesenchyme is bounded by a thin squamous mesothelium, which forms the lining of the exocoel. Between the sinus terminalis and the margin of the placenta there is a broad zone of chorion, also exposed to the uterine lumen. This is a thinner membrane which is almost non-vascular, being traversed only by a few small vessels which connect the sinus terminalis with the placental circulation. It is covered on the side next to the uterine lumen by a thin trophoblastic epithelium and it is lined, on the side next the exocoel, by the same squamous mesothelium as covers the inner surface of the yolk-sac splanchnopleur.

It is apparent that the direct approach to the foetal vitelline circulation from the uterine lumen involves traversing the yolk-sac endoderm first, then the basement membrane and mesenchyme surrounding the vessels and finally the endothelial walls of the vessels. Passage of substances from the uterine lumen into the exocoel would involve traversing either the endoderm, the basement membrane, the vascular mesenchyme and the mesothelium of the yolk-sac splanchnopleur, or alternatively the trophoblast, mesenchyme and mesothelium of the chorion. The amnion is separated from the uterine lumen by these membranes and by the fluid-filled cavity of the exocoel. The amnion is a very thin membrane, covered on the outside by the mesothelium lining the exocoel and lined on the inside by a thin squamous epithelium of ectoderm cells. By the 25th day of pregnancy the amount of exocoelomic fluid remaining, though variable, is usually small, so that often it was impossible to obtain a sufficient sample for testing. However, the considerable number of samples that were obtained did not vary greatly from the amniotic fluid in antibody

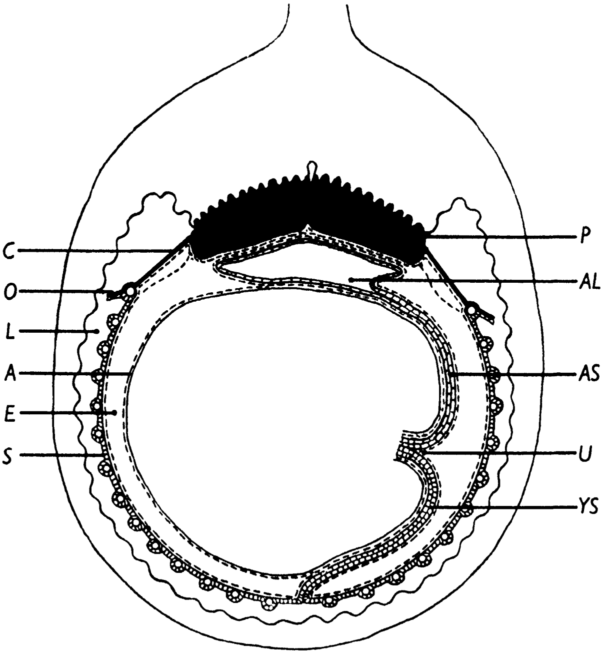

Fig. 1. Diagrammatic transverse section of the uterus and foetal membranes of a rabbit in late pregnancy. The foetus is omitted. Foetal ectoderm shown as continuous lines, foetal endoderm as cross-hatched, foetal mesoderm as broken lines. *A*, amnion; *AL*, allantois; *AS*, allantoic stalk; *C*, chorion; *E*, exoceol; *L*, uterine lumen; *O*, remnant of bilaminar omphalopleur; *P*, allanto-chorionic placenta; *S*, yolk-sac splanchnopleur; *U*, umbilicus; *YS*, yolk-sac stalk.

content. The latter could always be obtained in sufficient quantities at this stage, and it became the standard practice to collect the amniotic fluid and to neglect the exocoelomic fluid.

To study the possible entry of antibodies from the uterine lumen into the foetal vitelline circulation by the indirect route through the chorion and the exocoelomic surface of the yolk-sac splanchnopleur, thereby avoiding passage through the endoderm, immune serum was injected directly into the exocoel in a further series of experiments (Brambell, Hemmings, Hemmings, Henderson & Rowlands, 1951). Thus, in these, the outer surface of the amnion and the inner surface of the yolk-sac splanchnopleur were directly exposed to the immune serum.

It is worthy of emphasis that the arrangement of the foetal membranes in the rabbit is such as to render them peculiarly accessible to experiments involving exposure to solutions, since these can be injected so readily into the uterine lumen or even into the cavities enclosed by the membranes. It should be observed that passage through vascularized membranes, such as the yolk-sac splanchnopleur, presents a special problem. Passage could occur directly through such membranes, from one side to the other, as with non-vascular membranes, or passage could occur from one or other, or from both, sides into the blood vessels.

These are important considerations in relation to the results which are to be considered.

IV. EXPERIMENTAL RESULTS

Antibodies enter rapidly into the foetal circulation from immune rabbit serum injected into the uterine lumen. This also is the route by which antibodies enter from the maternal circulation. Provided that the quantity of immune serum injected is 0·5 ml. or over per embryo, the titre of foetal blood is related remarkably closely to the titre of the immune serum employed. This relationship holds whether the antibodies are bacterio-agglutinins, haemolysins, or antitoxins. The results of a series of experiments with agglutinins and haemolysins are shown in Table 1 as a correlation table of titre of foetal serum to titre of immune serum injected. It is evident that there is close correlation.

The same data are presented in Table 2 as distributions according to the ratio of the titre of the foetal serum to the titre of the immune serum injected; the data for agglutinins and haemolysins being shown separately. It is evident that, from the data available, there is no significant difference in behaviour between agglutinins and haemolysins, that in both the modal ratio is 1/16 and that the individual variation of foetuses is confined to two dilutions either side of the mode.

The data for diphtheria antitoxic sera are given as the ratio of the concentration of the foetal serum to the concentration of the immune serum injected in Table 6, column 2. Whereas the data presented in Tables 1 and 2 are based on the serum titres of individual foetuses, those in Table 6 are for the pooled sera of all the foetuses in a litter, individual variation being largely masked by pooling. It will be observed that the ratio conforms closely to the modal ratios for agglutinins and haemolysins.

Table 1. *Correlation of titre of foetal serum to titre of immune serum injected*

(Extracted from Brambell *et al.* 1949, 1950.)

Titre of serum injected	Titre of foetal serum						
	1/10	1/20	1/40	1/80	1/160	1/320	1/640
1/20,480	—	—	—	—	—	4	—
1/10,240	—	—	—	—	—	—	—
1/5,120	—	—	—	—	2	1	2
1/2,560	—	—	2	4	11	5	—
1/1,280	—	—	6	6	1	—	—
1/640	1	2	4	—	5	—	—
1/320	—	5	11	1	—	—	—

Table 2. *Titres of foetal sera expressed as ratios of the titres of the immune sera employed*

(Extracted from Brambell *et al.* 1949, 1950.)

Titre of foetal serum / Titre of serum injected	No. of foetuses observed	
	Agglutinins	Haemolysins
1/64	1	6
1/32	9	5
1/16	17	10
1/8	13	6
1/4	5	1
Total	45	28

The obvious route by which antibodies in the uterine lumen enter the foetal circulation in the area vasculosa is through the endoderm of the yolk-sac splanchnopleur. There is an alternative route through the chorion into the exocoel and thence through the mesothelium of the exocoelomic surface of the yolk-sac splanchnopleur. This would avoid traversing the endoderm. To distinguish between these, immune serum was injected directly into the exocoels of alternate embryos. Almost invariably some leaked back through the puncture into the uterine lumen. Hence the outer or endodermal surface of the yolk-sac splanchnopleur of all the embryos, both experimental and control, was exposed to the immune serum. In the experimental embryos the inner or exocoelomic surface also was exposed. The immune serum injected was diluted by the exocoelomic fluid. The results are given as the ratio of the titre of the foetal serum to the titre of the immune serum injected in Table 3. This ratio could not be calculated for those foetal sera which were negative at the lowest dilution of 1/10, but these were too few to invalidate the results. It can be seen from the close correspondence between the distributions of experimental and control foetuses that entry of antibodies into the foetal circulation must be mainly, if not entirely, through the outer surface of the yolk-sac and hence through the endoderm.

Table 3. *Titres of foetal sera expressed as ratios of the titres of immune sera injected into the exocoels*

(Extracted from Brambell *et al.* 1951.)

$\frac{\text{Titre of foetal serum}}{\text{Titre of serum injected}}$	Experimental foetuses	Control foetuses
Negative	3	4
1/128	1	2
1/64	10	10
1/32	17	10
1/16	9	3
1/8	3	3
1/4	—	1
Total	43	33

Comparison of the entry of antibodies prepared in cattle or horses with the entry of those prepared in rabbits shows that absorption into the foetal circulation is a selective process, the bovine or equine antibodies being transported in much lower degree than the rabbit antibodies. Antibrucella serum prepared in rabbits was injected into the lumen of one uterine horn and antibrucella serum prepared in cattle into the lumen of the other uterine horn in each of five rabbits pregnant 24 days. The titres of the foetal sera, expressed as ratios of the titres of the sera injected are shown in Table 4. In most cases the bovine antibodies could not be detected in the foetal serum even at the lowest dilutions, although the immune bovine sera employed were of higher titres than the immune rabbit sera. In those cases where bovine antibodies were detected in the foetal sera they were at much lower relative titres than the rabbit antibodies.

Similar experiments with antidiphtheria serum prepared in horses, the test for which is more sensitive, showed that the antitoxin invariably entered the foetal circulation but at concentrations of the same order as those of bovine agglutinins. It appeared possible that these results might be due to properties of the serum other than those of the antibody molecules themselves: for example, the foreign sera might be toxic or might block pores in the membrane, thus rendering it impermeable. On these grounds mixtures of immune rabbit serum with either immune bovine serum or immune equine serum were injected into the uterine lumen. The antibodies prepared in rabbits entered the foetal circulation as readily from such mixed sera as from pure rabbit serum, as is shown by the data in Table 5. The antibodies prepared in cattle or horses did not enter any more readily from such mixed sera than from pure sera.

Table 4. *Comparison of entry of antibrucella agglutinins prepared in rabbits and in cattle*

(Extracted from Brambell *et al.* 1950.)

Titre of foetal serum / Titre of serum injected	Foetuses exposed to rabbit antibodies	Foetuses exposed to bovine antibodies
1/256	—	3
1/128	—	3
1/64	—	—
1/32	1	—
1/16	4	—
1/8	1	—
1/4	5	—
Total: Positive	11	6
Negative	—	16

Table 5. *Entry of agglutinins or haemolysins prepared in rabbits from mixtures of rabbit and equine or bovine sera compared with that from pure rabbit sera*

(Extracted from Brambell *et al.* 1950.)

Titre of foetal serum / Titre of serum injected	Pure serum	Mixed serum
1/64	1	6
1/32	8	6
1/16	13	14
1/8	5	14
1/4	5	1

It was possible, consequently, to compare entry of antitoxins from mixtures of immune sera prepared in rabbits, cattle and horses, so long as each antiserum was prepared against a different antigen. It was necessary to pool the sera from all the foetuses in each litter to obtain quantities sufficient for all three tests. The results are shown in Table 6. It is evident that upwards of fifty times more antitoxin prepared in rabbits enters the foetal circulation than antitoxins prepared either in cattle or horses.

It may be concluded that the transport of antibodies from the uterine lumen into the foetal circulation is a selective process depending on some character of the antibody molecules peculiar to the species of animal in which they have been produced. It seems improbable that the molecular weight of the antibody is the determining factor. As is well known, these tend to fall into one of two size groups; those with molecular weight about 180,000, and those with molecular weight about 900,000. The rabbit agglutinins and the equine antitoxins belong to the group of smaller molecules, whereas the bovine agglutinins and the rabbit haemolysins belong to the group of larger molecules, a distribution that cuts across that of entry into the foetal circulation.

Table 6. *Entry of antitoxins prepared in rabbits, cattle and horses into the foetal circulation expressed as ratios of the concentrations of the mixture of immune sera employed*

(Extracted from Brambell *et al.* 1952.)

No. of experiment	Ratio: $\frac{\text{concentration of foetal serum}}{\text{concentration of serum injected}}$		
	Rabbit antitoxin	Bovine antitoxin	Equine antitoxin
569	*c.* 0·047	0·0002	—
613	0·059	0·0012	0·0013
578	0·077	< 0·0002	0·0009 to 0·0005
615	0·040	0·0008	< 0·0008

Results, as yet unpublished (Brambell, Hemmings and Oakley), obtained with antitoxins prepared in other species, show that the entry of these is intermediate between those for rabbit antitoxins on the one hand and bovine or equine antitoxins on the other hand.

There is evidence of another kind that the foetal yolk-sac splanchnopleur in rabbits is selective as between the different maternal serum protein fractions. Electrophoretic analyses of maternal and foetal sera in rabbits (Brambell, Hemmings, Henderson & Kekwick, 1953) have shown that the proportions of the electrophoretic fractions differ widely, the ratios of the foetal/maternal concentrations being for albumin *c.* 1/3, for α-globulin 2/3, for β-globulin 3/2, and for γ-globulin *c.* 2/5. This suggests a differential selection of these protein fractions by the foetal membrane, which we hope to investigate by using radioactive isotopes as markers.

Estimations of the antitoxin content of the tissues of the yolk-sac splanchnopleur after exposure to a mixture of antitoxic sera should provide a useful clue to the mechanism of selection. Such determinations of the antitoxic content of the foetal membranes were made on material from the experiments referred to in Table 6 and from two other animals, exposed for shorter periods of 3 and 6 hr. respectively. The results are given in Table 7.

Table 7. *Relative concentrations of antitoxins in other foetal fluids and in the tissues of the membrane*

(Extracted from Brambell *et al.* 1952.)

No.	569	613	578	615	902	903
Duration of exposure (hr.) ...	24	22	24	23	6	3
Rabbit/bovine:						
Washings	0·97	1·51	2·83	0·99	1·50	1·23
Splanchnopleur	< 2·26	2·72	2·19	1·59	1·09	1·27
Amniotic fluid	2·24	1·30	1·78	1·48	1·56	—
Stomach contents	—	1·30	1·87	3·66	—	—
Rabbit/equine:						
Washings	—	1·07	2·04	0·86	1·34	1·15
Splanchnopleur	—	1·86	1·62	1·29	1·00	1·00
Amniotic fluid	—	1·25	1·34	1·12	1·12	—
Stomach contents	—	1·14	1·58	1·84	—	—

It might be supposed that if the bovine and equine antitoxins were prevented from entering the membrane, then the relative concentrations in the tissues would be similar to those in the foetal circulation, the rabbit antitoxin preponderating greatly. If all were admitted equally freely by the endoderm, and exclusion of the heterologous antitoxins was effected by the vascular endothelium, the only other continuous cellular layer separating the circulation from the uterine lumen, then a large excess of bovine and equine antitoxins might be built up in the tissues around the vessels. Neither of these expectations was realized. The ratios observed varied from unity to 2·72. Such determinations are, however, liable to error from two sources, first from antitoxin adhering to the outer surface of the membrane (although this was well washed in saline), and secondly, from residual blood remaining in the vessels. Although they were drained, they were not transfused, and it can be calculated that 4 % of blood in the tissues would be sufficient to account for the small but significant excess of rabbit antitoxin observed in the tissues. These results are not conclusive, and it would be unwise to place too much reliance upon them.

Antibodies enter the amniotic fluid from the uterine lumen also. The absolute concentrations attained in the amniotic fluid may not be of much significance, since the volume of fluid in the amnion is very variable, but the relative concentrations of antibodies prepared in different species are interesting. The ratios for antitoxins, in the four experiments already referred to in Table 6, are given in Table 7. It will be apparent that whereas rabbit antitoxin enters the amniotic fluid much less freely than the foetal circulation, the reverse is true of bovine and equine antitoxins. Consequently, the relative entry of rabbit/bovine and rabbit/equine antitoxins into the amniotic fluid only slightly, though significantly, exceeds unity. This suggests that entry takes place directly through the membranes from the uterine lumen and not indirectly by way of the foetal circulation. The nearly uniform entry of rabbit, bovine and equine antitoxins into the amniotic fluid does not justify the assumption that it is due to an active process of selective absorption and secretion on the part of the cells, such as is required to account for the entry of rabbit antitoxins into the circulation. It may be of an entirely different character not involving cellular activity. Since the concentrations attained within the amnion after 24 hr. exposure are of the order of less than 1/100 of those without, it is evident that an effective barrier to free passage does exist. The gradual passage through this barrier, in slightly different amounts for each kind of antitoxin, could be accounted for by purely physical forces. It may be that antibodies 'seep' into the amniotic cavity between the cells of the multicellular membrane, perhaps even through local faults that must occur in such membranes. Since the entry of bovine and equine antibodies into the foetal circulation is of the same order as into the amniotic fluid, it is possible that it may be due to 'seepage' between the cells.

Antibodies are found also in the stomach contents of foetuses which have been exposed to immune serum. These appear to be derived from the amniotic fluid, but often are more concentrated in the stomach than in the amnion. The ratios are given in Table 7.

Finally, attempts were made to recover the residues of the serum injected from the uterine lumina at the conclusions of the experiments. Too little fluid remained to collect, so saline washings were taken. The results are included in Table 7. The value for no. 569 was obtained from saline transfusion of the intact uterus. The other values refer to saline in which the excised foetal yolk-sac splanchnopleurs were washed. It is probable that minute quantities of foetal blood remaining in these membranes may have contaminated the latter group and may account for the tendency for the ratios to be above unity.

V. DISCUSSION

The mammalian foetus provides an excellent approach to the problem of the relation of the plasma protein reserve to the tissue protein. In some species maternal plasma protein reaches the foetus in the form of antibody. Possibly maternal plasma protein, of which the antibody is a conveniently recognizable fraction, may play normally an important part in foetal metabolism. Certainly, in some cases, the antibody may cause serious damage to the foetus.

Technically it is not easy to determine the quantity of protein transported across unit area of the maternal/foetal barrier in unit time, and, so far, we have not attempted to do so. What has been measured is the concentration of antibody built up in the foetal serum, a quantity not necessarily related to specific rate of transport in any simple fashion. It would be premature to attempt a coherent hypothesis of molecular exchange across this barrier on the evidence available, but some discussion of the problems involved appears justified. The reasons for regarding the appearance of the specific antibody in the foetal circulation as evidence of the transport of the molecules through the foetal membranes substantially unaltered, have been discussed already.

The embryonic membranes are multicellular and complex. It would be quite misleading to treat them as though they were simple membranes of uniform structure. First, being multicellular, transport through them could occur either between or through the component cells. Individual cells must die, and may leave, temporarily, actual gaps in the membrane. Openings may form due to changes of shape and movements of the cells. The intercellular substance between the cells may be permeable to protein. It is difficult to conceive a mechanism adequate to explain the large entry of protein through such a membrane, which is capable at the same time of retaining the foetal fluids, unless it be by active transport by the cells. The cells need not all be in the same physiological state; the sum of their cell membranes cannot have the uniformity of the cell membrane of a single cell at any given moment. Active transport of large molecules by a cell could take place by ingestion in droplets of fluid and their transport in vacuoles or by their attachment to and passage through the cell membrane into the organized substance of the protoplasm. It is worth noting that whereas the former process could be selective only in an all-or-none fashion of accepting certain fluids and rejecting others, the latter process could be selective also in the sense of accepting certain molecular species while rejecting others presented to it simultaneously in a common substance.

It was found that bovine and equine antibodies occur in the foetal sera at nearly the same concentrations, relative to each other, as in the mixed sera injected. They are both at much lower concentration relative to rabbit antibodies. This led to the suggestion that the difference might be due to qualitatively different methods of entry into the foetal circulation (Brambell *et al.* 1952). It was suggested that the entry of the foreign antibodies might be due to a process of slow 'seepage' between the cells of the multicellular membrane. This would not necessarily involve an active process of absorption and secretion on the part of the cells such as the rapid entry of rabbit antibodies appeared to imply. However, more recent work, still in progress (Brambell, Hemmings & Oakley, unpublished) shows that antibodies prepared in certain other species appear in the foetal circulation in concentrations intermediate between the low bovine and equine values and the high rabbit ones. In this respect it appears now that antibodies prepared from a range of species form a graded series rather than two clearly defined groups.

The selective effect could be produced in any one of three ways, or in any combination of them. First, by differential destruction of the antibodies before reaching the barrier between the uterine lumen and the foetal circulation. Secondly, by differential destruction after passing the barrier. Thirdly, by selection at the barrier. The first two hypotheses involve differential destruction of antibodies, such as occurs when foreign proteins are administered parenterally to the adult organism. In the non-immune animal the removal is gradual while the antigenic properties of the foreign antibody globulin result in its rapid removal through serological reactions in the immune adult. No satisfactory evidence has been obtained so far of such destruction of the foreign antibodies, either in the uterine lumen or in the foetus during the course of the experiments. Some contrary evidence has been forthcoming. Material recovered from the uterine lumen, either by perfusion or by washing the foetal membranes at the conclusion of the experiments, was found to have ratios of rabbit to bovine or equine antibodies not greatly exceeding those in the mixed serum injected. Moreover, the ratios in the tissues of the splanchnopleur, in the amniotic fluid and in the stomach contents were similar (Table 7). If differential destruction in the uterine lumen, before reaching the barrier, accounted for the ratio in the foetal circulation, the ratios in the other foetal fluids and tissues should be similar to that in the foetal serum. This is strong evidence that destruction in the uterine lumen alone cannot account for the results.

Destruction of the foreign protein after passing the barrier would need to be exceedingly rapid to account for the ratios observed. Serological reactions scarcely can be invoked since the mammalian foetus does not appear to be capable of producing such antibodies itself, so far as is known at present. No clear evidence of destruction has been obtained. Bovine and equine antibodies are found in the foetal circulation for at least 96 hr. and the concentrations after 48 hr. are not much lower than after 24 hr. from the time of injection into the uterine lumen. Although this could be accounted for by continued uptake rather than persistence of the foreign antibodies, it does not provide any positive evidence of rapid destruction.

Although the hypothesis of differential destruction after passing the barrier cannot be excluded, that of selection at the barrier appears preferable at present.

Wherever selection occurs, the means by which it is brought about is a problem of no less interest. A selective transport favouring certain molecular species at the expense of others in the same substrate would appear to be a very strong indication of a process involving at some point an intimate relation with the organized structure of the protoplasm. It is not possible to separate the corresponding fractions from mixed sera by any known physical means, and immunological methods must be employed to effect such separation *in vitro*. Hence it may be suspected that the mechanism of separation *in vivo* is immunological in character, using the term in a broad sense, and that it involves something in the nature of the union of antibody with antigen or of enzyme with substrate. It must be remembered that the rabbits employed had not been immunized previously, and that the 24 hr. duration of the exposure to the foreign proteins during the experiments was too short to permit of the development in the mother of any known kind of immunity.

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TRANSPORT OF LIPID THROUGH CELL MEMBRANES

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I. INTRODUCTION

The metabolism of lipids in the animal body involves their absorption from the intestinal lumen, distribution in the body, uptake by and release from adipose tissue and utilization for energy purposes in the liver and other cells. The passage of lipids into the intestinal cell, the adipose tissue cell and the liver cell and the transport of lipid material in the blood between the intestine, the fat stores and the liver are facts that are generally accepted by physiologists. The object of this paper is to consider in detail the evidence for the transport of lipids into these three groups of cells in the living body, to examine the type of lipid involved in this transfer and the factors that appear to influence it in each case.

Types of lipid molecules

The term 'lipid' is applied to a group of substances with certain common lipoid characteristics that are frequently associated together in biological systems. The most important lipids are:

(i) *Fatty acids*

(*a*) *Saturated series.* This comprises acetic acid up to fatty acids containing 30 carbons or more. Natural fatty acids usually contain an even number of carbons; those with 10 carbons or less are volatile in steam and are liquid at mammalian body temperature. The commonest natural saturated fatty acids are lauric, myristic, palmitic and stearic acids.

(*b*) *Unsaturated series.* This includes the most common of all natural fatty acids, oleic acid; this is liquid at mammalian body temperature. There are also several polyethenoid acids. Those containing the grouping $—CH:CH.CH_2.CH:CH—$ are dietary essentials since this configuration cannot be synthesized in the body.

(ii) *Glycerides*

Fatty acids occur as mono-, di- and tri-esters with glycerol, the latter being the commonest form in the body.

(iii) *Phospholipids and cerebrosides*

Lecithins and cephalins are glyceride esters containing fatty acids, phosphoric acid and a base. Plasmalogens are similar but contain fatty aldehydes instead of fatty acids. Carbohydrate containing phospholipids and a non-glyceride phospholipid, sphingomyelin, have also been isolated. Cerebrosides contain fatty acid, carbohydrate and base, and are closely related to the sphingomyelins. The most important phospholipid for consideration in connexion with lipid transport would seem to be lecithin.

(iv) *Sterols*

Many sterols occur in the body and have various pharmacological actions. So far as lipid transport is concerned, cholesterol appears to be the most important.

II. THE INTESTINAL CELL

(i) *Structure of the cell*

The absorbing unit in the small intestine is a columnar cell with a thick striated outer border next to the intestinal lumen. This outer border has been shown by Baker (1942) to be penetrated by fine canals about 0·3μ in diameter. Electron microscope studies (Granger & Baker, 1950) have revealed a fine fibrilliform structure of the outer border—the fibrils running in the same axis as these canals. The canals can be shown to be filled with lipid-staining material only during the absorption of fat (Baker, 1951). Between the outer border of the cell and the nucleus is the Golgi organ; this part of the cell appears to be rich in phospholipid. The cell contains a number of enzymes including alkaline phosphatase.

(ii) *Evidence for transport of lipids through the intestinal cell*

Numerous observers have noted that glycerides disappear from the intestinal lumen and simultaneously lipid-staining material can be demonstrated inside the intestinal cells while the chyle becomes laden with fat. This situation persists just as long as there is lipid being absorbed from the intestinal lumen. Although the intestinal cell may synthesize glycerides, visible lipid cannot be demonstrated in the intestinal cells or the chyle during carbohydrate or protein absorption. Labelled glyceride molecules have been traced through the cell into the chyle from the intestinal lumen. Lecithin can be readily traced into the intestinal cells, and labelled cholesterol was found to pass from the intestinal lumen with fat into the chyle (Biggs, Friedman & Byers, 1951). It has been claimed that plant sterols are not absorbed (Schoenheimer, 1931), but the evidence does not preclude their destruction in the intestinal cell.

There seems little doubt that lipids pass through the intestinal cell during absorption. The cell is an active metabolic unit capable of modifying, or even synthesizing, many types of lipid molecule, so that comparison of the lipid material found in the intestinal lumen with that recovered from the chyle is not a reliable indication of selective permeability of the intestinal membranes.

(iii) *Forms of lipid transported*

For nearly half a century it has been thought that lipids had to be reduced to some water-soluble form before absorption could occur (Verzár & McDougall, 1936; Bloor, 1943). In the case of glycerides this was said to be achieved by complete hydrolysis and solubilization of the liberated fatty acids by the hydrotropic action of bile salts. On the basis of considerable experimental evidence to the contrary, this concept of complete hydrolysis has been challenged (Frazer, 1938, 1946, 1952*a*). That hydrolysis is partial and not complete has recently been conclusively proved by studies using labelled materials (Favarger & Collet, 1949; Favarger, Collet & Cherbuliez, 1951; Karnovsky & Gidez, 1951; Reiser, Bryson, Carr & Kuiken, 1952; Borgström, 1952).

Partial hydrolysis results in the presentation of a water-insoluble as well as a water-soluble lipid fraction to the intestinal cell for transport. This water-insoluble portion is present in the intestinal lumen as a finely dispersed oil-in-water emulsion of glycerides containing other lipid-soluble molecules. It can be shown that fine emulsification is an essential step in the absorption of the glyceride fraction (Frazer, Schulman & Stewart, 1944; Daniel, Frazer, French & Sammons, 1951; Frazer, 1952*b*). Phospholipid is not hydrolysed in the intestinal lumen in rats or human subjects (Frazer, Sagrott & Sammons, 1949). Cholesterol appears to be absorbed in the glyceride fraction. The water-soluble lipid fraction presumably enters the cell in ionic or molecular form.

(iv) *Factors concerned in lipid transport through the intestinal cell*

It would appear that the first essential factor determining whether the lipid molecule will pass in the water-soluble or water-insoluble fraction is its partition coefficient between oil and water. Thus, the lipid molecules are 'partitioned' into two groups in the intestinal lumen. Normally the water-soluble component consists essentially of the shorter chain, and perhaps some of the more unsaturated, fatty acids. Long chain, and more saturated, fatty acids tend to remain in the oil phase. This partition of absorbed fatty material originally predicted from relatively indirect evidence (Frazer, 1948*a*) has now been conclusively proved using ^{14}C-labelled fatty acids (Bloom, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Chaikoff, Bloom, Stevens, Reinhardt & Dauben, 1951; Bloom, Chaikoff & Reinhardt, 1951; Kiyasu, Bloom & Chaikoff, 1953). The water-soluble fatty acids are mainly absorbed by diffusion, but there is not sufficient evidence to show whether any selective transfer of these molecules occurs. The absorption of tributyrin and fatty acids is not affected by double adrenalectomy in rats (Bavetta & Deuel, 1942; Frazer, 1948*b*).

Particulate absorption of the water-insoluble glycerides occurs mainly in the upper intestine. The particles must be less than 0·5μ in diameter and negatively charged for absorption to occur (Frazer *et al.* 1944). Particulate absorption is entirely prevented by faulty intraluminar emulsification, but in pancreatic enzyme deficiency it can be re-established by the intraduodenal administration of finely emulsified fat (Frazer, 1952*c*). Glyceride absorption is depressed by double adrenalectomy in rats (Verzár & Laszt, 1934, 1935; Barnes Miller & Burr, 1941), but facilitated by the simultaneous administration of choline, glycerophosphate or lecithin (Frazer, 1951). Particulate absorption is depressed in human subjects in the sprue syndrome possibly due to excessive mucus secretion and decreased intestinal motility. Pumping of the villi and water flow may be important factors (Frazer, 1952*d*).

It would therefore appear that the most important form of lipid transported through the intestinal cell is glyceride in particulate form. These glyceride particles may contain other lipid-soluble molecules such as cholesterol. Those lipids that can be removed from the oil into the water phase may pass into the cell by diffusion.

III. TRANSPORT OF LIPID IN BLOOD AND LYMPH

(i) *Structure of plasma*

The resting blood plasma contains 400–500 mg. of lipid per 100 ml. of plasma, which is mainly in the form of phospholipid and cholesterol. The phospholipid is largely lecithin containing palmitic, oleic and linoleic acids, while 60% of the cholesterol is esterified with more unsaturated fatty acids. The level of cholesterol is affected by age (Keys, Mickelsen, Miller, Hayes & Todd, 1950), by the dietary level of fat (Keys, 1952) and by endocrine factors.

These lipids are associated with protein to form macro-molecules. Two plasma lipoproteins have been characterized—an α-lipoprotein that is about 300 Å. long and 50 Å. wide and has a molecular weight of about 200,000; and a β-lipoprotein that is spherical with a diameter of about 185 Å. and molecular weight about 1,300,000. Some 75% of the lipid in fasting plasma can be accounted for as β-lipoprotein (Gurd, Oncley, Edsall & Cohn, 1949).

After fat feeding and under certain other conditions there is a marked increase in blood lipids. This normally takes the form of an increase of the glyceride fraction which is commonly accompanied by a concomitant smaller increase of phospholipid and cholesterol. The glyceride is in particulate form and causes a marked turbidity of the plasma. This emulsion of glycerides remains stable in the plasma for long periods. Stability appears to depend upon lecithin since destruction of the lecithin by D-lecithinase causes clumping and creaming of the chylomicrons (Frazer, Elkes, Sammons, Govan & Cooke, 1945). The phospholipid appears to link the glyceride particles to globulin so that they display certain reactions characteristic of this protein fraction (Elkes, Frazer & Stewart, 1939).

(ii) *Evidence for lipid transport in the blood and types of lipid involved*

Lipid transport in the blood is proved if lipid can be shown to be added to the blood at one point and removed at another. This is the case with glycerides which are injected into the blood stream from the thoracic duct and subsequently removed into adipose tissue or the liver and other cells. Labelled glyceride has been traced from the intestinal lumen to these different destinations. Turn-over of plasma phospholipids has been demonstrated, and it was suggested that glycerides might be converted into lecithin in the liver in preparation for transport to, and metabolism by, the extrahepatic tissues. Recent work shows that this is not the case and that the liver is the main site of both formation and removal of plasma lecithin (Entenman, Chaikoff & Zilversmit, 1946). Furthermore, extrahepatic tissues can readily use glycerides or fatty acids (Geyer, Cunningham & Pendergast, 1950; Goldman, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Geyer & Cunningham, 1950), and can synthesize phospholipids *in situ* if they are required. Similarly, there is little reason to believe that cholesterol plays a major part in the transport of other lipids in the blood.

(iii) *Factors affecting lipid transport*

Little is known about factors controlling lipoprotein structure and stability. There is some evidence that abnormal lipoproteins can occur under certain circumstances. An abnormal and unstable lipoprotein fraction has been demonstrated in ageing subjects and may be responsible for deposition of lipid in the blood vessel walls (Gofman, Jones, Lindgren, Lyon, Elliott & Strisower, 1950). Whether lipoprotein structure varies significantly under normal physiological conditions, other than with ageing, remains to be seen.

With regard to particulate glyceride, the importance of the integrity of the stabilizing lecithin has already been noted. It has also been shown that heparin can abolish the abnormal turbidity of alimentary hyperlipaemia. This was said to be due to finer dispersion of the glyceride (Hahn, 1943; Weld, 1944, 1946), but recent work indicates that heparin may be more concerned with the removal of fat from the blood stream than dispersion of the lipid in the plasma (Brown, 1952). While the question must be left open at present, there is no doubt that the effect of heparins and antiheparins must be taken into account in any consideration of lipid transport in the blood.

(iv) *Transfer to and from the blood and lymph*

Negatively charged particles appear to pass more readily from the tissue spaces into lymphatics than into blood capillaries. The reasons for this apparent selectivity are not clear. In the small intestine the negatively charged particles of glyceride enter the lacteal rather than the capillaries and so pass almost exclusively into the chyle. The particulate fat is transferred from the lymph into the blood stream by 'intravenous injection' via the thoracic duct. Water-soluble lipids, such as short-chain fatty acids, may pass into the capillaries and be conveyed in the portal blood.

The particulate glycerides pass out of the blood to cells where they may be stored or metabolized. How this outward passage of particulate fat occurs is still a mystery. Certain factors appear to control this removal—the possible effect of heparins and antiheparins has already been mentioned. In occasional subjects the rate of removal is slowed so that progressive accumulation of particulate glyceride in the blood follows normal fat intake and the blood fat may exceed 10%. The level of blood glycerides rapidly decreases if fat is excluded from the diet (Holt, Aylward & Timbres, 1939; Lawrence, 1946; Stanley & Thannhauser, 1949).

It may, therefore, be concluded that the only certain evidence of lipid transport in the blood at the present time is that concerned with the conveyance of glycerides in particulate form from the intestine to the adipose tissue and from these fat stores to the liver and elsewhere. The other lipid components of plasma appear to be essentially structural and turn-over of lecithin and cholesterol mainly reflects metabolic activity in the liver.

IV. ADIPOSE TISSUE CELLS

(i) *Structure*

Adipose tissue cells are macrophages that are particularly concerned with the assimilation of fat from, and subsequent release into, the blood and tissue spaces. The detailed mechanism of this uptake and mobilization of fat is unknown. Adipose tissue cells can also synthesize fats and glycogen (Tuerkischer & Wertheimer, 1942). Special collections of adipose tissue—so-called 'brown fat'—occur in hibernating animals and seem to have more extensive functions (Wendt, 1937; Eger, 1938). When fully developed, adipose tissue cells appear as large nucleated cells in which the whole cytoplasm appears to be replaced by triglyceride fat.

(ii) *Evidence of lipid transport into and out of adipose tissue cells*

As already mentioned, adipose tissue cells can synthesize glycerides from non-lipid material. However, labelled glycerides have been traced from the intestine into the fat depots and thence back into the blood stream to the liver. The half-life of the fat depots in the rat was found to be 6–8 days (Schoenheimer & Rittenberg, 1936). When hyperlipaemic blood circulated through the capillaries, there was a marked reduction in glyceride content as shown by simultaneous arterial and venous analyses. There would seem to be no doubt, therefore, that lipids can pass into the adipose tissue cell, be retained in the cell for storage purposes and mobilized again into the blood stream ultimately to be used by the tissues.

(iii) *Form of lipid transported*

The lipid in the adipose tissue cells consists of triglyceride esters of long-chain fatty acids. There is a close similarity between the depot fats and dietary fat provided that there is a reasonably high level of fat in the diet (Hilditch, 1947). The selection of the more saturated fats for deposition in the fat depots can be explained on the basis of the Partition Hypothesis of fat absorption—synthesized fats also tend to belong to the saturated series. Hilditch & Stainsby (1935) have suggested that biohydrogenation may occur. In any case, the lipid is presented to the intestinal cell as fine particles of triglyceride. There is no evidence at present to indicate whether these particles of fat undergo any preparatory changes before entry into the adipose tissue cell. Although esterases occur in most tissues, there is no evidence of a significant degree of true lipase activity in adipose tissue—the introduction of a potent lipase into such tissues causes classical 'fat necrosis'.

The adipose tissue cells are continually releasing glycerides into the circulation, so that a fairly rapid turn-over of the fat occurs. The mobilization of the depot fat causes a visible hyperlipaemia consisting of a marked increase of particulate glycerides. By what means, and in what form, this lipid passes from the adipose tissue cells is not known.

(iv) *Factors concerned in deposition and mobilization of fat from adipose tissue cells*

The main factor that determines the accumulation of fat in fat depots is the relationship of supply to demand. Thus appetite greater than that required to meet the energy demand results in surplus intake of carbohydrate and fat, both of which may give rise to increased deposition of glyceride in the adipose tissue cells. As already mentioned, heparins and antiheparins may play some unknown part in the mechanism of removal of particulate fat from the blood stream. The major cause of obesity is probably excessive synthesis of fat from non-lipid sources; this synthesis is stimulated by insulin (Renold, Marble and Fawcett, 1950; Scott & Engel, 1950).

Various factors are alleged to enhance the mobilization of fat from the adipose tissue cells. An endogenous hyperlipaemia occurs in starvation but to what extent this is an unmasking of normal turn-over which is obscured by alimentary hyperlipaemia is not known. A more definite fat mobilization seems to follow the administration of lipogenic hormones of the anterior part of the pituitary gland (Stetten & Salcedo, 1945; Campbell & Lucas, 1951). Adrenalectomy in rats is said to alter the mobilization of fat from adipose tissue (Stoerk & Porter, 1950). Nervous lesions (Wertheimer, 1926) also influence the rate of mobilization from the affected area. Fenton & Carr (1951) demonstrated genetic factors—yellow mice becoming obese while their non-yellow litter-mates did not, although consuming the same synthetic diet.

V. LIVER CELLS

(i) *Structure*

The liver cell is enclosed in a membrane that comes into intimate contact with the blood stream. Lipids enter into the structure of the liver cell: the mitochondria may contain more than 25% of lipid, mostly phospholipid containing arachidonic acid. These highly unsaturated fatty acids do not appear to undergo oxidative degradation and may be protected by their close association with protein (Bensley, 1937; Kennedy & Lehninger, 1949). The integrity of the liver cell is important for phospholipid and cholesterol synthesis (Ada, 1944; Hevesy, 1945; Bloch, Borek & Rittenberg, 1946).

(ii) *Evidence for lipid transport into and out of the liver cell and types of lipid involved*

It can be readily demonstrated that intact or isolated liver preparations utilize labelled fatty acids or glycerides. This metabolism of lipids seems to occur inside the cell and, if it is interfered with, intracellular accumulation of lipid may occur. It seems clear that lipid can readily pass from the outside to the inside of the liver cell. Does lipid also pass in the other direction? Some lipids, such as phospholipid and cholesterol, must pass from inside the liver cell into the blood since the turn-over of these molecules in the plasma is a reflexion of turn-over in the liver. Glyceride, on the other hand, does not normally pass out from the liver cell again but is utilized. It was at one time thought that long-chain saturated fatty acids were desaturated in the liver and that the unsaturated fatty acids formed were transported to the extrahepatic tissues for combustion. It is now clear, however, that the most unsaturated fatty acids in the liver are neither oxidized nor transported away from the liver, that desaturation is not a necessary preliminary to utilization of fats by the liver cells or extrahepatic tissues, and that saturated fats can be utilized by the extrahepatic tissues in the absence of the liver. In choline deficiency fats accumulate in the liver; this has been attributed to faulty phospholipid formation (McArthur, Lucas & Best, 1947), and it has been suggested that this prevents the transport of the fat away from the liver cells. Hartroft (1951) has demonstrated the accumulation and removal of fat in the choline deficient liver in a series of dramatic histological preparations. While his interpretation may be correct under the abnormal conditions of gross fat accumulation observed in choline deficiency, it can be shown that transport of lecithin away from the liver is not an effective form of expenditure of liver glycerides. Choline deficiency does not impair the ability of the glycerides to enter the liver cells, but it probably interferes in some way with the utilization of glycerides within the cell. Thus the available evidence supports the view that glycerides can be transported into, but not out of, the liver cells except as degradation products.

(iii) *Factors affecting liver lipid*

The essential factor affecting the glyceride in the liver cells is the relationship of supply and demand. If large quantities of lipid come to the liver, as in starvation or after a heavy fatty meal, accumulation of glyceride may occur. Alternatively, interference with the rate of utilization of glycerides, as in poisoning or choline deficiency, may also cause a significant increase in intracellular and extracellular glyceride. The origin of this lipid from the diet or fat depots can be checked by the use of labelled fats. Endocrine factors control the synthesis of lipids by the liver, insulin favouring fatty acid and cortisone cholesterol synthesis. Thus, marked changes in liver lipids that are not concerned in any way with lipid transport can occur.

During recent years much attention has been paid to phospholipids and cholesterol as possible forms of transportable lipid. Emphasis has been largely placed on formation of some water-soluble and diffusible compound or complex. It is becoming increasingly apparent that this is not the case, and that the most important lipid component in fat transport during intestinal absorption, or in the blood, or for storage in adipose tissue, or for metabolic use by the liver and extrahepatic tissues, is glyceride fat in particulate form. Phospholipid plays an important part in cell structure; it is essential to the stability of particulate glyceride in the blood and may represent a vital step in the metabolism of glycerides in the liver cell, but it does not appear to be significantly used as an intermediate in lipid transport. Cholesterol also has structural functions, and it can be slowly transported to the tissues, but it is not an important factor in fatty acid transport. The passage of finely dispersed, negatively charged fat particles through membranes raises new problems, many of which cannot be solved until certain basic information becomes available.

VI. SUMMARY

1. Since hydrolysis of glycerides is incomplete in the intestinal lumen, lipids are 'partitioned' before absorption into water-soluble and water-insoluble fractions.

2. The water-insoluble glyceride fraction enters and leaves the intestinal cell in particulate form.

3. Particulate fat passes preferentially into the lacteals while the water-soluble lipid may enter the blood capillaries direct.

4. The main form of lipid transported in the blood stream is particulate triglyceride. Phospholipids and cholesterols are only removed extremely slowly by the extrahepatic tissues. The turn-over of lecithin and cholesterol in the plasma is a reflexion of turn-over in the liver.

5. Particulate glyceride is removed from the blood stream into adipose tissue. Heparins and antiheparins may play an important part in the removal of particulate fat from the blood.

6. Fat is mobilized from the fat depots and appears in the blood as triglyceride in particulate form. The mechanism of mobilization is obscure.

7. The liver takes up particulate glycerides and metabolizes them. There is no adequate evidence of fatty acids or glycerides passing out from the liver cells. Phospholipids and cholesterol synthesized in the liver cells can pass into the blood stream, but the liver is the main site for their removal from the blood.

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MORPHOLOGICAL AND MOLECULAR ASPECTS OF ACTIVE TRANSPORT

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I. INTRODUCTION

In the majority of papers contributed to this Symposium the morphological and molecular aspects of active transport and secretion have been somewhat neglected, although the necessity for paying attention to these aspects was emphasized in the introductory papers of Ramsay and of Davson, and in the paper by Frazer. To some extent contributors to the discussion, particularly V. B. Wigglesworth, have emphasized the importance of paying more attention to the morphology of cells and membranes involved in active transport. But for the most part the papers have accepted as their starting point that active transport occurs, and have concentrated on studies of kinetics of transfer, upon the linkage of transport with metabolism and upon the facts revealed, for example, by the use of competing molecules and of enzyme poisons. This paper will concentrate on the two aspects, morphological and molecular, which have been relatively neglected.

II. ANALYSIS OF ACTIVE TRANSFER AT DIFFERENT MORPHOLOGICAL LEVELS

There are at least six levels at which it is profitable to consider active transfers. The first level is that of the simplest known cellular membranes, such as those of red blood cells, where morphological complications are minimal. At this level we encounter processes ranging from *simple diffusion* under thermal agitation, through *facilitated diffusion*, up to the full mechanism of *active transfer*. Thus with human red cells ethyl alcohol appears to pass through the membrane by simple diffusion, glycerol and glucose by facilitated diffusion and simple diffusion, sodium and potassium by active transport and simple diffusion. Electron-microscope studies have not so far given any indication that the red cell membrane is at all complicated morphologically. At present simple diffusion processes have a satisfactory quantitative theory, theories are being developed for facilitated diffusion, and active transport is largely lacking a theory.

The second level is that of the cell membranes which are specialized for the performance of active transport, such as the membranes of intestinal epithelial cells, kidney tubule cells and motor end-plates. These specialized structures are often very rich indeed in particular enzymes, e.g. alkaline phosphatase at the surface of intestinal epithelium and proximal tubule cells, choline esterase at the surface of motor end-plate cells. In A. C. Frazer's paper in this volume, particular attention is directed to the probability that the structure of the free border of the intestinal epithelium is critical in fat transport. However, in general we have at present very little knowledge of the real functions of these specialized cell membranes. It has been suggested that the high concentrations of enzymes in these specialized membranes are concerned in active transport by serving as the centres through which chemical energy is transformed into mechanical energy, just as adenosine triphosphatase acts as the centre through which the chemical energy of adenosine triphosphate is transformed into the mechanical energy for contraction of muscle actomyosin (Danielli, 1951, 1952, 1953, 1954). Recently Sjostrand & Rhodin (1953) have shown by electron microscopy that the apical zone of kidney proximal tubule cells is a system of fine ducts, about 600 Å. in diameter, thus resembling the apical zone of intestinal epithelial cells, but having ducts which are smaller in diameter. It seems possible that an exploration of the function of enzymes in relation to this type of structure would be richly rewarding.

The third level is that of mitochondria. The work of Davies and his colleagues (reported in this Symposium) shows that mitochondria have a remarkable ability to transport ions across their membranes. Sjostrand (1953) and Sjostrand & Rhodin (1953) have revealed to a remarkable degree the complex membrane structure of mitochondria, but it has not so far been possible to relate the physiological function of mitochondria to their structure and enzyme organization, so far as the field of active transport is concerned. Sjostrand & Rhodin have also made many suggestive observations on the relationship between mitochondria and cellular membranes. Unfortunately, much of this work has been on osmium tetroxide fixed material rather than on frozen-dried material, so it is uncertain how far these observations have physiological significance. Some incidental observations which I have made on the mitochondria of frozen-dried sections suggest that mitochondria may at times protrude into the lumen of a tubule or become part of the cell membrane. At present we do not know, for example, how mitochondria participate in ionic regulation in a mammalian kidney. It would be very much easier to understand how this occurred if we knew that mitochondria project into the lumen of a tubule or, as Wigglesworth suggests, absorb material from the lumen by being applied to the base of the apical ducts.

The fourth level of approach is to vacuoles. What is the significance, from the point of view of active transfer, of those vacuoles which include among their properties the ability to concentrate neutral red? Are the osmotic and regulative properties of contractile vacuoles to be ascribed solely to their associated mitochondria, or have the vacuoles intrinsic regulative activity?

The fifth level of approach is to pinocytosis,* the phenomenon of absorption of environmental fluid as discrete droplets by undulating membranes, first observed by W. H. Lewis. Anyone who has seen a film showing the flood of vacuoles passing across a cell as a result of pinocytosis will readily realize that any theory of kinetics of penetration through cell membranes must break down utterly if applied to a cell which can display pinocytosis.

The sixth approach is to discover the complete physiological significance of extracellular processes, such as lymph flows, capillary flows and pumping activities such as those displayed by intestinal villi.

A complete understanding of active transport in an organ will only be possible when we can give an integrated account of the functions of all the processes concerned with active transport which occur at these six morphologically distinct levels. In making the analysis which will permit such an integrated account we must recognize both the hierarchy of morphological units discussed above and a hierarchy of processes which may occur as part of the functioning of each morphological unit. This hierarchy of processes includes (*a*) simple diffusion, (*b*) facilitated diffusion and (*c*) active transport. Simple diffusion, i.e. movement of molecules under the driving force of thermal agitation, unrestricted by steric factors and unlimited by molecular structure, can be handled quantitatively. Facilitated diffusion cannot yet be treated quantitatively, and has been recognized as a process occurring at a limited fraction of the cell surface (at so-called active patches), only since quantitative treatment of simple diffusion through cell membranes was possible (Danielli, 1943). Facilitated diffusion occurs under the driving force of thermal agitation, and the equilibrium reached is the same as that achieved by simple diffusion. But facilitated diffusion at any one site, unlike simple diffusion, is restricted both by structural and steric factors so that only a small number of molecular species are concerned. For example, a hexose penetrates cell membranes by simple diffusion about 10^3–10^4 times more slowly than does ethanol. But at a site of facilitated diffusion specific for certain hexoses, these hexoses will penetrate at a rate comparable to ethanol, i.e. 10^3–10^4 times faster than by simple diffusion. For the moment we may contrast facilitated diffusion with active transport by defining active transport processes as those in which molecules are transferred across membranes by the use of an energy supply other than, or additional to,

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* At the Symposium a film of pinocytosis, lent by A. Hughes, was shown at this point.

thermal agitation. But it is wisest to regard these definitions as of transient value, for, on the one hand, as our knowledge increases it may become necessary to subdivide what we now call 'active transport', and, on the other hand, as we shall see later, some conceivable types of facilitated diffusion may require energy for maintaining structural units, including carriers or expanded lattices, although the actual movement of a molecule from one side of a membrane to the other may proceed under thermal agitation only. Thus there may not be a sharp division between all forms of facilitated diffusion, and all forms of active transfer, so far as energy requirements are concerned.

It seems to me better to adopt this analytical approach to active transport, and recognize that some of our definitions are of transient value, rather than to adopt the method, which Rosenberg has advocated in this Symposium, of attempting to find a rigorous all-inclusive definition of active transport, for the latter method is handicapped by emphasis on energy differences between initial and final states, to the exclusion of the processes intervening between these states. For example, a cellular membrane may well transfer sodium chloride solution from one side to the other of the membrane, say by a vacuolar process. Thermodynamically, the initial and final states on the two sides of the membrane are indistinguishable, and thus by Rosenberg's definition the process is not active transfer. But since vacuolar processes require energy, to a biologist this transfer is active. Thermodynamically it would be recognized as active transport if the free energy of the whole system (including the membrane) was assessed. But this, in the majority of cases, would be impossibly difficult, since it is usually impracticable to measure experimentally what part of the free-energy change occurring in a cellular membrane is associated with active transfer rather than with other cellular processes.

III. THE RELATIONSHIPS OF ENZYMES AND ENZYME POISONS TO TRANSFER

That certain substances which are enzyme poisons inhibit transfer processes is certain; how these substances act is a matter of conjecture. Outstanding examples of such action include the inhibition of glucose transfer in intestinal epithelial cells by iodoacetate (Wilbrandt & Laszt, 1933); of glucose transfer in kidney proximal tubules by phloridzin (Nakazawa, 1922); of facilitated diffusion of glycerol in red cells by copper (Jacobs & Corson, 1934), by SH reagents (LeFevre, 1948); of facilitated diffusion of glucose in red cells by phloretin phosphate (Wilbrandt & Rosenberg, 1950), by dinitrofluorobenzene and by diazonium hydroxides (Bowyer, 1953); of active transfer of sodium and potassium in red cells by eserine (Holland

& Grieg, 1950) and by diazonium hydroxides (Bowyer, 1953). But it does not follow that these substances necessarily act by poisoning enzymes.

Enzymes, by definition, are cellular catalysts, i.e. enzymes are substances found in (or synthesized by) cells, which accelerate chemical reactions but do not modify chemical equilibrium conditions. Since not all cellular processes are chemical, it is improbable that all accelerated biological processes are normally accelerated by enzymes. Precise evidence is required before we can decide whether a process is enzymic or not. To illustrate this necessity we may consider generalized cell permeability: at one time, because permeations had temperature coefficients of the same order of magnitude as those found for chemical reactions, it was supposed that all permeations involved chemical reactions (and must be enzymic). This rather widely held theory was based almost entirely on this one point of resemblance between permeations and chemical reactions. But a more detailed analysis showed that this resemblance was misleading; any process in which a resistance, which may be represented by an activation energy, is overcome directly by thermal agitation will be exponentially related to temperature. Chemical reactions are one such process; permeations of cell membranes by simple diffusion are another. Hence these processes show similar temperature coefficients (Danielli, 1934). This example makes explicit the danger of concluding that two processes are identical because they have a few quantitative or qualitative similarities. Facilitated diffusion and enzymic processes have several such similarities: they are inhibited by similar substances; structural and steric properties are of importance in both cases; the processes have similar temperature coefficients. But such resemblances are no proof of identity. In 1939 I was able to show, by analysis of the kinetic studies of Jacobs, that glycerol penetrates the red cells of certain species by two processes, one of which was simple diffusion and the other involved a selective activity of a small fraction (less than 1 %) of the surface area of the cells. These active patches in many ways resembled enzymes, and this resemblance was strengthened by Davson's (1942) studies. But there is still no proof available that enzymes are in fact involved. Proof could perhaps be obtained by inhibiting a facilitated diffusion process with an irreversible reagent such as dinitrofluorobenzene, followed by a demonstration of a strict parallelism between enzyme inhibition and inhibition of facilitated diffusion, backed up by demonstration that enzyme isolated in purified condition from inhibited cells was in fact combined with the inhibitor. At present we lack evidence of this quality for a single case.

On the other hand, if we make a short list of enzymic processes which may be involved *indirectly* in facilitated diffusion and active transfer, we at once see that many enzymes may be concerned and that many substances may poison them, so stopping transfer, and yet none of them may be involved in the direct process of transfer. Enzymic processes of interest in active transport include:

(*a*) *Enzymes providing energy for transfer*, e.g. all enzymes concerned in adenosine triphosphate synthesis are of interest if ATP is the basic energy source. Phosphokinase inhibitors, or substances like dinitrophenol which break the linkage between phosphate uptake and oxidation, will inhibit transfers based on ATP, without necessity for a direct action on the process of transfer.

(*b*) *Enzymes with a trapping function.* Once a substance has entered a cell its accumulation may be assisted by conversion to a derivative, e.g. glucose and inorganic phosphate may be accumulated as a less diffusible glucose phosphate. If glucose is the penetrating species, the conversion may be effected by ATP, catalysed by a hexokinase. If phosphate is the penetrating species, it may be trapped by formation starch or glycogen catalysed by a phosphorylase, or taken up in an enzymically catalysed phosphorylative oxidation. In both cases the enzyme has a trapping function and accumulation will be inhibited if the enzyme is inhibited.

(*c*) *Enzymes with a maintenance function.* It is possible that the active regions of a cell membrane are unstable and require 'servicing' by enzymes. For example, enzymic phosphorylation of an unstable carrier molecule may be required, or the active form of the membrane may be an expanded lattice structure which collapses into a more stable structure and requires energy for re-expansion. The servicing enzymes in such cases would not participate directly in transfer, and there would probably be no stoichiometric relationship between maintenance energy and number of molecules transferred.

(*d*) *Enzymes forming or emptying vacuoles.* In addition to any enzymes which are concerned with the transfer of substances into vacuoles, there may be other enzymes concerned in the movement and discharge of vacuoles, actuating contractile proteins, as is found with the ATPase-actomyosin complex. Inhibition of such enzymes will also inhibit over-all active transfer.

(*e*) *Enzymes participating directly in active transfer.* The contractile protein theory of Goldacre (1952), as developed by Danielli (1951, 1952, 1953, 1954), is concerned with one type of mechanism whereby an enzymic centre is an integral part of a true active transfer mechanism. It is only in cases where an enzyme poison acts upon mechanisms of this type that we learn anything of the actual mechanism of transfer across a membrane.

It seems likely that enzymes which are concerned with actuating or servicing the direct mechanisms of transfer will vary from cell to cell, and also with the type of molecule to be transferred, for such variation will permit transfers of different molecules to occur independently (Danielli, 1954). It is of interest in this connexion that the evidence at present available suggests that phosphatases are concerned in the transfer of glucose across the membranes of red cells, intestinal epithelial cells and kidney proximal tubule cells, whereas choline esterase is concerned with transfer of sodium and potassium across red cell membranes and motor end-plate membranes.

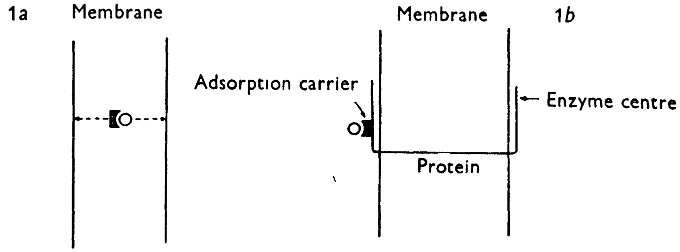
IV. THE IMMEDIATE MOLECULAR MECHANISM OF FACILITATED DIFFUSION AND ACTIVE TRANSFER

The plasma membrane of most cells appears to consist basically of a lipoid layer about 50Å. in thickness (roughly bimolecular) with protein layers on either side. Movement of molecules across such membranes is limited mainly by hydrogen-bond formation between water and the molecules concerned, and by the effective viscous and structural resistance of the membrane. In the main, facilitated diffusion is concerned with the accelerated transfer of molecules which penetrate only slowly by simple diffusion because of extensive hydrogen-bond formation, and active transport is concerned primarily with accelerated transfer and establishment of concentration gradients of the same molecular species. Both facilitated diffusion and active transport must occur within the limits laid down by the size and structure of the membrane, and the central problem is probably that of overcoming the hydrogen-bond limitation, for there is little evidence so far that reducing the viscous and structural resistances of the membrane are ever of importance except in so far as these factors also are involved in the hydrogen-bond problem. There appear at present to be two alternative approaches to this central problem: the first from the point of view of macromolecular processes of the membrane and the second from the point of view of the molecular process acting upon the transferred molecule.

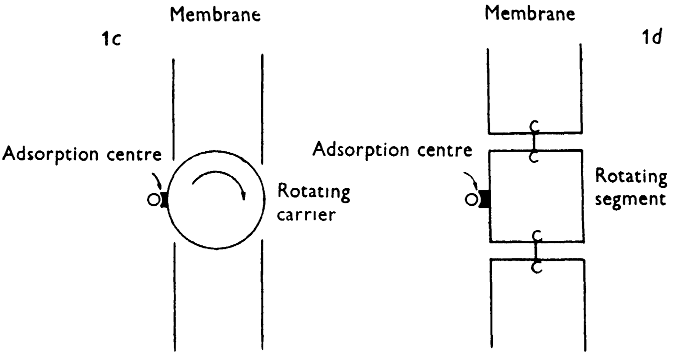
Macromolecular membrane processes

(*a*) The simplest process which might have validity is the diffusing shuttle or carrier molecule, to which Osterhaut has given much attention (Fig. 1 *a*). The essentials of such a molecule are that it should be relatively insoluble in water and readily dissolved in lipoid, should not itself form many hydrogen bonds with water, should readily and reversibly form a complex with the molecule to be transferred which also will not form many hydrogen bonds with water, and further that both the free carrier and the complex should diffuse readily in the lipoid layer. If these criteria were satisfied, the carrier molecules, while executing the random movement of thermal agitation, would effectively shuttle between the two sides of the membrane. Such mechanisms will accelerate the attainment of equilibrium, and so may be classified as facilitated diffusion. Wilbrandt & Rosenberg (1952) have discussed such a system in relation to glucose transport.

(*b*) A second mechanism is that of the propelled shuttle (Fig. 1*b*). In this mechanism an enzyme centre such as phosphatase or choline esterase supplies energy to actuate a contractile protein. The contractile protein has an adsorption centre which will be on one side of the membrane when the protein is contracted and on the other side when the protein is extended. The properties required of the adsorption centre are similar to those required for the carrier molecule in mechanism (*a*). According to the details of the contraction and expansion, such a process could merely accelerate the attainment of equilibrium, or cause the building-up of concentration gradients. This mechanism has been discussed in more detail elsewhere (Danielli, 1952, 1953, 1954).



Diffusing shuttle or carrier Propelled shuttle or carrier



Rotating molecular carrier Rotating molecular segment carrier

Fig. 1.

(*c*) A third mechanism is that of a rotating molecular carrier (Fig. 1*c*). A molecule of diameter equal to the thickness of the plasma membrane will rotate very rapidly in a medium of the viscosity of water, and even in a medium of the apparent viscosity of the plasma membrane (about 10^5 times that of water) the rate of rotation will be high. Some special properties would be required to retain the molecule in the membrane, such as inability to form hydrogen bonds with water. And it would need to have a special adsorption centre with the same properties as the adsorption centre in mechanism (*b*), or the carrier in mechanism (*a*). With such properties a rotating carrier would provide a mechanism for facilitated diffusion.

(*d*) The concept of a rotating carrier molecule presents certain mechanical and stability problems which would perhaps be more readily resolved if the rotating unit were not a whole molecule, but a molecular segment (Fig. 1*d*). A molecular segment such as that shown in the figure would be able to rotate about an axis composed of two covalent bonds, such as carbon-carbon, carbon-oxygen, or carbon-nitrogen. The energy required for rotating about such bonds is of the order of 3000 cal., so that the average energy required to rotate a segment would be 6000 cal., and the proportion of such segments rotating at any time would be proportional to $e^{-6000/RT}$. The order of magnitude of the average rate of rotation would be given by $kT = Iw^2$, where I is the moment of inertia and w the angular velocity of the segment. An adsorption centre would be required as in mechanism (*c*).

It is not quite inconceivable that the rotating units in mechanisms (*c*) and (*d*) could be energized by enzymic centres, so that the heat of reaction at the enzyme centre gave additional rotational energy to the unit.

(*e*) A fifth quite different mechanism can be based upon the idea of expanded lattices. If the structure of the lattice of say a protein in the membrane were expanded, molecules of the correct size and structure would be able to penetrate into and through the lattice, and thus through the membrane. This would constitute facilitated diffusion. The energy for this expansion might be provided by enzyme action. F. Bowyer has pointed out to me that if such a lattice expanded and collapsed asymmetrically, it would constitute a pump imparting one-way transfer to those species able to enter the expanded lattice.

The five types of macromolecular process envisaged above, with their variants, provide possible mechanisms, the energy requirements of which range from zero in some cases, through a 'servicing' energy requirement in other cases to further cases in which the energy requirement would be stoichiometrically related to the rate of transfer. These mechanisms have in common the necessity for an adsorption centre (or lattice) which is specific for a limited range of molecules. The forces available for adsorption with most molecules of biological interest appear at first sight to be limited to Van der Waals forces, electrostatic forces and hydrogen-bond forces (Danielli, 1944), which are not specific in themselves. Consequently specificity must reside in the distribution of these forces in space. However, exactly the same physical problem arises in the determination of the specificity of enzymes. It therefore seems quite possible, for example, that a substance which will inhibit the hydrolysis and synthesis of glucose phosphate by phosphatase will also interact with the specific adsorption centres of transfer mechanisms for glucose or phosphate, and so inhibit these also. Thus at this stage of the analysis it appears that inhibition of a specific transfer process by a specific enzyme poison is not good evidence that the enzyme for which the poison is specific is part of the intimate mechanism of transfer.

The molecular process acting upon the transferred molecule

Turning now to consider the molecules which are transferred, our attention is immediately focused upon the mechanisms whereby hydrogen bonding between these molecules and water may be broken. Specific adsorption upon a carrier unit will be almost useless in all the mechanisms described above (except possibly the expanded lattice, mechanism (*e*)) unless the number of hydrogen bonds is reduced to a maximum of two. In the case of glucose, for example, which forms five hydrogen bonds with water, the activation energy for permeation into cells by simple diffusion is of the order of 15,000–25,000 cal. With an activation energy as high as this permeation is very slow. When glucose is penetrating into human or rabbit red cells by facilitated diffusion, the activation energy is reduced to about 4000 cal., so that four out of the five bonds have been broken by some process other than thermal agitation, and penetration occurs at about the same rate as is found for methyl alcohol, which also forms only one hydrogen bond with water.

Some insight into possible mechanisms may be gained by considering methods of denaturing proteins. The structure of proteins is largely maintained by hydrogen bonds; these bonds are broken by adding protons (as hydrogen ions) or by adding alternative hydrogen-bonding agents (e.g. urea). It is reasonable to suppose that these two methods will also break hydrogen bonds with water. Thus we conclude that for maximum efficiency the specific adsorption centres in carrier molecules should provide hydrogen-bonding groups and should be proton conductors so that protons may be readily available. Proteins, nucleic acids and probably polysaccharides will be able to act as hydrogen-bond formers, and in so far as these compounds are themselves hydrogen-bonded, or rich in groups such as COOH, they will be proton conductors. On the other hand, the hydrocarbon moiety of lipoids would be quite unable to form hydrogen bonds or conduct protons.

Whilst the ideal adsorption centre is a hydrogen-bond forming proton conductor, its selectivity will be determined by its organization in space, and here the distribution of hydrogen-bond forming groups may be of particular importance. We now know that native proteins are often not homogeneous in their structure, but consist of lamellae, each of which is composed of one or more polypeptide chains. In each lamella the polypeptide chains are oriented so that the polar side-chains are in one surface and the non-polar side-chains are in the opposite surface. In a soluble protein these lamellae are grouped in pairs, with their non-polar surfaces together, and with polar surfaces either exposed to water or facing a polar surface of another pair of lamellae. Where polar surfaces of lamellae are in contact with one another they may adhere strongly, as is the case, for example, in haemoglobin, where the pairs of lamellae may be dissociated by urea. Since such surfaces adhere so strongly it is possible that units based on protein lamellae may extend right through the membrane, as indicated in Fig. 2*a*. With the arrangement of lamellae of Fig. 2*a* a hydrogen-bonding channel or slit will exist right through the membrane (which will otherwise be lipoid in character). Such channels may contain much water, as is the case with native proteins in crystals. But in so far as such channels exist they must constitute a small fraction of the cell surface, and must be too tightly bonded to permit entry of the great majority even of small molecules. If this were not so we should not find the quantitative relationship between oil-water partition coefficients and rates of penetration, which has been established for many years. For example, if these hydrogen-bonding channels were equivalent to aqueous channels, methyl alcohol and glycerol should have the same activation energy of permeation and should penetrate at almost the same rate (according to the equation $PM^{\frac{1}{2}} = \text{constant}$). In fact, the activation energies for permeation of these molecules are normally about 4000 and 12,000 cal. respectively, and methyl alcohol permeates at least 100 times faster than does glycerol. Slow permeation for glycerol is often coexistent with rapid facilitated diffusion of other molecules such as amino-acids and sugars. On the other hand, Collander has shown that in some cells, although the great majority of molecules obey the quantitative relationships expected for molecules diffusing through a lipoid layer, the smallest molecules, such as formamide and methyl alcohol, may enter cells more rapidly than is calculated, which he explains in terms of minute pores in the membrane. Similarly, Jacobs (1952) and Ussing (p. 409) have found anomalies in the rate of penetration of water which they explain in terms of pores. It is possible that the hydrogen-bonding channels indicated in Fig. 2*a* would permit the rather indiscriminate passage of such very small molecules, but would be quite impermeable to other molecules unless these molecules had a specific structure.

Returning to Fig. 2*c*, we see that a polypeptide chain or lamella, forming part of a hydrogen-bonding channel, may extend on to the outer and inner surfaces of the lipoid part of the membrane, and thus could constitute the active unit in a propelled shuttle or carrier (mechanism (*b*), Fig. 1*b*). Thus a system such as Fig. 2*c*, if not energized, would permit selective facilitated diffusion through its hydrogen-bonding channels, and if energized would provide active transport and a means of building up concentration gradients.

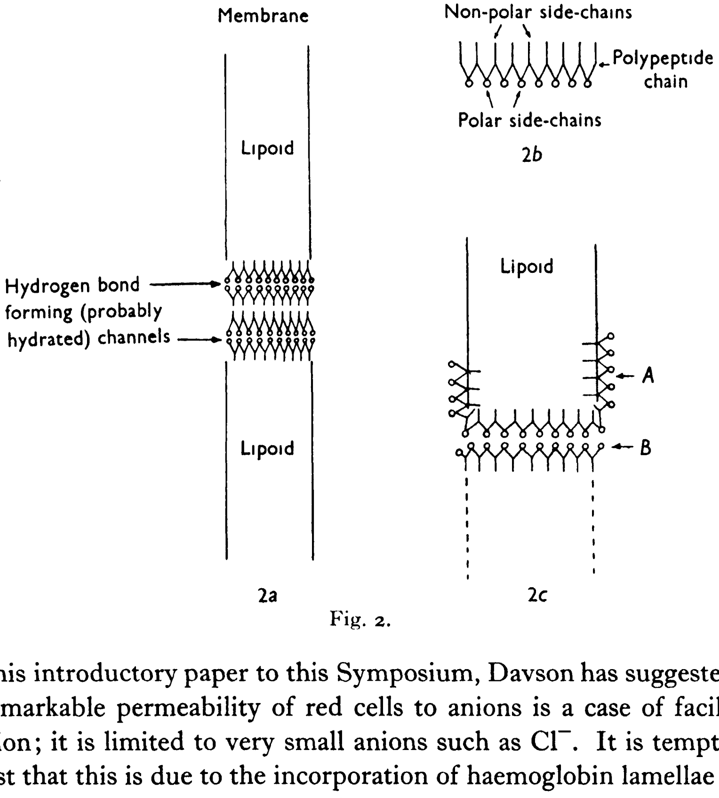


Fig. 2.

In his introductory paper to this Symposium, Davson has suggested that the remarkable permeability of red cells to anions is a case of facilitated diffusion; it is limited to very small anions such as Cl^-. It is tempting to suggest that this is due to the incorporation of haemoglobin lamellae in the membrane, thus providing positively charged hydrogen-bonding channels which will facilitate the diffusion of small anions.

Other characteristics of proton-conducting hydrogen-bonding systems

The synthesis and hydrolysis of esters, acetals, glycosides and peptides are catalysed by protons (supplied as hydrogen ions). It might therefore be suspected that a proton-conducting hydrogen-bonding system would be catalytic. This consideration reminded me that S. J. Holt had drawn my attention to the catalytic effect of ion exchange resins. Cation-exchanging resins are of necessity proton conductors, and although their surfaces, unlike those of proteins, cannot be expected to be highly specific towards reactants, some catalytic effect might nevertheless be expected. Papers by Sussman (1946) and by Underwood & Deatherage (1952) show that cation-exchanging resins are in fact catalytic for hydrolysis and synthesis of a wide variety of compounds, including hydrolysis of proteins. A paper by Thomas & Davies (1947) shows that resins are more effective catalysts than can be explained by their hydrogen-ion content. By comparison with hydrogen ions in bulk solution, the hydrogen ions of resins are more effective by the following factors:

1·7-fold for hydrolysis of methyl acetate,
2·3-fold for hydrolysis of ethyl acetate,
9·8-fold for hydrolysis of butyl acetate.

The order of increasing efficiency is the same as the order of increasing adsorption of the ester. Consequently it appears that in addition to the catalytic effect of the protons of the resin, the resin surface is contributing a second catalytic influence.

From these facts it is apparent that if we specify that the active membrane unit in facilitated diffusion or active transport is a stereochemically specific proton-conducting hydrogen-bonding system, we are probably also specifying that this system shall have catalytic activity. Indeed, it may well be that all enzymes of the great family which catalyse synthesis and hydrolysis of esters, glycosides, peptides, etc., owe their activity to being proton-conducting hydrogen-bonding systems whose stereochemistry determines which reactions they may catalyse. The active membrane units in facilitated diffusion and active transport may be members of this family in the same sense that the haemoglobins are members of the family of enzymes which owe their activities to the properties of the ion-porphyrin complex. If the active membrane units are catalytic, fresh possibilities arise for their selectivity; they may select molecules from the environment by reversible exchange reactions. For example, an amino-acid may be selected from the environment by an exhange reaction with a peptide chain of the active unit, thus:

$$\text{polypeptide} - \mathrm{CO.NH.CH}R\mathrm{.CO.NH} - \text{polypeptide} + \mathrm{NH_2.CH}R^1\mathrm{.COOH},$$
$$\downarrow$$
$$\text{polypeptide} - \mathrm{CO.NH.CH}R^1\mathrm{.COOH} + \mathrm{NH_2CH}R\mathrm{.CO.NH} - \text{polypeptide.}$$

And after transfer to the far side of the membrane an exchange reaction in the reverse direction would release the amino-acid. A similar process could be involved in transport of sugars, etc.

It may well be that polynucleotides and polysaccharides can be integral parts of such processes, in addition to proteins. Some polynucleotides have recently been found to have peptidase activity, and the structure of nucleic acids is now believed to be maintained by hydrogen bonding. Neuberg & Roberts (1949) and Lansing & Rosenthal (1952) have obtained evidence suggesting that polynucleotides are concerned in active transport.

V. CONCLUSION

It is apparent that physico-chemical considerations provide a wealth of possibilities which need further consideration; many of these arise from Goldacre's theory of the function of contractile proteins. It is possible also that studies of active transfer have brought us to the point where we must reconsider the cellular function of enzymes. Cellular enzymes may be not merely catalysts which facilitate certain reactions but polyfunctional centres whose function in one context may be chemical, in another mechanical, perhaps sometimes both. So far as active transport is concerned it seems clear that future work on any biological system involves:

(1) Analysis of the number of morphological units involved, as outlined in § II of this paper.

(2) Analysis of the number of distinct processes involved in each morphological unit, as outlined in §§ II and III.

(3) Analysis of the molecular mechanism of each process, as outlined in § IV.

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