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PREFACE

This volume contains the papers read at a Symposium of the Society for Experimental Biology held at Leeds in September 1954. It is the ninth of an annual series of reports.

The meeting at Leeds was held in the University at the invitation of the Vice-Chancellor and the Society wishes to record its gratitude for this courtesy. Generous help was given by many members of the University during the course of the meeting and to them the Society acknowledges its indebtedness.

Financial support for the meeting was provided by the Rockefeller Foundation, the British Council, Messrs. Imperial Chemical Industries Ltd. and Messrs. Courtaulds Ltd. Without this help, for which the Society is deeply grateful, the meeting could not have been held.

The Editors wish to thank the Cambridge University Press for considerable help in the preparation of this volume.

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

May 1955

INTRODUCTORY REVIEW

By W. T. ASTBURY
Department of Biomolecular Structure, University of Leeds

I rather think that the only symposium of this kind more important than the one we are holding now would be entitled 'The Proteins', and that simply because it would be about the same things—the 'first things'—but more comprehensive. The proteins, being organized systems of polypeptide chains, though in combination sometimes with prosthetic groups, are all fibrous in a crypto-sense, and it is partly a convenience to restrict ourselves, as we are doing on this occasion, to the so-called 'fibrous proteins', meaning thereby those that are more obviously so, that do not wander about much, and are found in fact to serve mainly covering, framework and mechanical purposes. We cannot draw the line sharply, and the chief reason for this meeting and choice of programme is that it is with the fibrous proteins that we are at the moment getting on best—so well, we believe, that there is intensified research activity and mounting excitement about them everywhere. This symposium is a sign of the times, and most definitely what the history books used to call 'a good thing'. If we do not make a memorable showing, it will not be through lack of a worthy topic.

The programme has been subdivided in a way that is of course not the only one possible, and I do not propose to review it piecemeal but only to indicate the trends, so as to orient ourselves effectively with respect to all the detail that must follow. First comes a glance at 'straight' (but not straightforward) chemical analysis, the principal 'trend' that underlies everything else and is with us always. All that we ask, at least for a start, is still no more than what amino-acid residues are present in the polypeptide chains, and in what order! Sanger will tell us whether the answer is very much nearer; and he should know, having just completed his analysis of the only protein molecule, insulin, ever yet worked out in full. This is not to forget the great quantities of more diffuse chemical information that continue to be accumulated and that we shall need to keep on sifting all the time, off and on. So often, besides, the proteins operate in inseparable combination not only with prosthetic groups but with other macromolecules, such as the polysaccharides and nucleic acids, hardly less formidable than themselves; and to the nucleic acids and nucleoproteins in particular, if we are to achieve any worth-while understanding of the molecular basis of reproduction, it appears increasingly urgent to devote the closest

attention, much more than we are doing at present. In this field the problem of specificity comes to a head, so to speak, and it is stimulating, if not altogether convincing, to find that some people are now inclined to ascribe it all ultimately to the distribution of nucleotides in the nucleic acids rather than to that of the amino-acid residues in the protein chains. We may look to Chargaff to tell us more about these things. He it is who has been chiefly responsible for the recent demonstration of systematic nucleotide differences among the nucleic acids which in turn has given rise to the newer attempts to define the detailed shape of the nucleic acid molecule, with the view, of course, of relating it eventually to that of its specially chosen opposite number (or precursor?), the polypeptide chain.

This question of detailed molecular shape must obviously run through all our deliberations in one way or another; in the end it is the paramount consideration. We are still only groping as regards the corpuscular proteins, but as I have just said, the fibrous proteins are at last becoming distinctly encouraging. It all began, I suppose, with the realization through X-ray studies that there are so few fundamental configurational types—perhaps only two, broadly speaking, that we have come to call the keratin-myosin-epidermin-fibrinogen group (k-m-e-f group for short), and the collagen group. As biological tissues have evolved, there have been many variations on these two major themes or master plans—variations which the correct answer must accommodate automatically, for they are an essential part of the problem—and we are bound to explore these ramifications too to some extent, and against what we can make out of their common metabolic background (Rudall's and Neuberger's papers, for example, come in here); but all the same it will be gratifying enough if during the next few days we succeed in clarifying merely the outlines of the master plans themselves. Thanks largely to the ideas of Pauling and Corey and especially their application to the structure of synthetic polypeptides, the molecular configurations characteristic of the k-m-e-f group are now assuming outlines to inspire a fair amount of confidence, but the situation as regards the collagen configuration remains much more debatable, and we shall expect the M.I.T. and King's College, London, schools in particular to debate it hard. The issue is still almost entirely one of X-ray interpretation—we know of no other comprehensive test for the k-m-e-f and collagen groups than the large-angle X-ray diagrams which are the origin of the classification, though naturally chemical ones have been proposed and are being looked for all the time—and it will no doubt follow the prevailing fashion of invoking various helical arrangements of the polypeptide chains. The important discovery made by Randall and his collaborators that the collagen chains can be stretched by something like 10%, thereby improving the X-ray pattern

enormously (though not transforming it into anything fundamentally different), should now prove a powerful aid in deciding which form of helix, if any. I personally cannot help thinking that there can scarcely be much of a helix, otherwise there would be greater and easier extensibility; whereas, of course, it is an outstanding biological function of tendons and the like *not* to 'give'. For many years there has been a quest, in which we at Leeds have frequently taken part, for some means of transforming members of the collagen group, in the way the members of the k-m-e-f group can be transformed, into an extended β-configuration, but the only hint of possible success so far comes from an experiment carried out by Rudall. He reported several years ago that the earthworm cuticle (e.g. raised in boric acid, cleaned in trypsin, washed, and finally stretched) gives an excellent X-ray diagram of the collagen type. The picture shows no signs of β-form, but when the same preparation, originally soluble in warm water, is treated for a couple of weeks with formic acid (about 90 %), the resulting solution dries down to a film insoluble in boiling water which Ruddall now finds (after further treating it with 5 % acetic acid for 20 hr. and washing in order to remove any remaining normal collagen) gives a well-defined β-diagram. Unfortunately, it turns out to be a disoriented diagram even when the X-ray beam is directed parallel to the film, but there is no mistaking what it is. The experiment is far from conclusive because it may have served only to concentrate some hitherto unrecognized component of the cuticle, but the indications are against that, and in my view it holds out considerable promise. Best of all, nevertheless, would be to find a means, if such there be, of transforming the collagen configuration into the β-configuration simply by stretching. It is crucial to know whether or not this can be done, and if it cannot, any model worthy of the name must tell us why at once.

Talking about the classification of the fibrous proteins in terms of X-ray diagrams and molecular configurations stirs rather a metaphysical thought that may be worth spending a few moments with in passing. The two great k-m-e-f and collagen families of fibrous proteins were so named to express or imply a concept in molecular biology, as a result of looking for some of the molecular roots of the large-scale family tree of orthodox biology; for underlying the phylogeny of visible structures there must be a corresponding, and in a sense more clear-cut, lineage of the fundamental biological macromolecules, and particularly of the proteins. So there was a peculiar satisfaction and thrill in the recognition that the feared multitude of different protein fibres amounted in the end to so few really different types, and it was natural for a time for the biological significance of the generalization to be uppermost in one's mind. A similar viewpoint focused attention on the subdivision into long-range elastic structures (the k-m-e-f group)

and relatively inextensible structures (the collagen group), and it was seen also that the intramolecular transformations of the former from the normal folded α-configuration to the extended β-configuration or to the 'super-contracted' state offered an interpretation not only of the more familiar muscular activity but of the principal manifestations of biological elasticity and movement in general. Formally, the supreme molecular biological significance of all lies in the universal exploitation in nature of the properties of long-chain molecules as such, with their endless capacity for change of shape, for instance, from which derives ultimately the characteristic flexibility and stretchability of all biological structures; but here in the k-m-e-f and collagen groups was something more, something more 'planned' in the way of chain configurations; and especially in the k-m-e-f group did we encounter not simply the common random folding of long chains, but a comprehensive system of polypeptides folded in definite configurations for definite purposes.

There followed then many attempts to elucidate the α- and β-configurations (and the collagen configuration) by every physical, chemical and crystallographic means that could be brought to bear on them, culminating, as just mentioned, in the Pauling and Corey interpretation, which has now given us pretty convincingly, among other more controversial proposals (e.g. for the collagen group), the so-called 3·6 α-helix as the basis of the normal unstretched form of the k-m-e-f group, and various 'pleated sheets' for the extended β-form. The derivation of these two fundamental types has been clinched finally from studies of the structures of synthetic polypeptides (using chiefly preparations and experimental data obtained in the Courtaulds Research Laboratory—see, in a similar connexion, Malcolm's infra-red contribution to the muscle section of our symposium), and the resulting tendency, strengthened by certain indications that the α-configuration may take part also in the structures of corpuscular proteins, is thus to look at them now rather through physico-chemical than biological eyes—to think of them, that is, merely as energetically favoured forms of the polypeptide chain, and to talk of α- and β-proteins without reference to possible biological correlations. A tentative molecular phylogeny seems for the present to have been obscured behind a purely physico-chemical classification.

The whimsical thought prompted by these events, perfectly reasonable though they are, is a re-emergence in molecular guise of that old riddle: which came first, the chicken or the egg? I am not of course putting this question for serious discussion or arguing some distinction or precedence as between biological and chemical evolution, but I do feel that this nursery reminder makes a point here that is not entirely out of place. I mean that

beyond our structural studies of the biological macromolecules there still remains the profounder background of their growth and developmental relationships—how it all came about—and I should like to make a plea for not letting this aspect slip too much out of sight. The great task of unification, and the founding thereon of a new, deeper and simpler biology, is still practically untouched.

The title of this symposium is 'Fibrous Proteins *and their Biological Significance*', and if I interpret it correctly, it is an invitation to advance along just those lines. We are offered papers, for example, on flagella, cilia and muscle, which surely comprise a single molecular topic if ever there was one. The muscle part of the story we have long accepted as telling of one of the sovereign biological activities that has inspired a host of investigators. It continues from week to week as excitingly as ever—and still with the solution 'just round the corner'; but recently it has been recognized that the time has come for abating a little our excessive preoccupation with muscle proper and reorienting some of our attention towards other, and conceivably more primitive and direct, manifestations of vital mechanics. At and below the level of the microscope there is a hectic underworld of beating, lashing, spiralling and what-not organelles of dimensions reaching right down to the macromolecular, and they are endowed with systematic properties of motility every bit as significant and representative as those of the comparatively gigantic muscle apparatus; probably, in fact, we may venture further and infer that they are activated by the self-same principles, when these are reduced to their essentials. As my colleagues and I will describe later is this symposium, the gap between the extremes of muscles and bacterial flagella seems to have been bridged in one stroke by the discovery that their X-ray diagrams are so closely related that not only do they both belong to the k-m-e-f group but also they have very likely the same axial macro-period of about 410 Å. And what is more, the composite nature of the bacterial flagella diagram, indicating the simultaneous presence of *two* configurations of polypeptide chains, the α and the supercontracted, suggests at once how the rhythmic bending processes may be brought about, namely, by the propagation of waves of folding along selected chains.

We have experiments in progress, too, to see what can be found out by X-rays and supporting techniques about the molecular structure of the larger flagella—those of algae, spermatozoa and similar minute organisms equipped with motile appendages. Meanwhile, in this particular range, there has come to light as the integrated result of electron-microscope studies all over the world—Bradfield will review these and bring us up to date with regard to the wide explorations he himself has made—a wonderful

generalization that to me, and no doubt many more, stands out as one of the most important micro-anatomical revelations of our time. I refer, of course, to the discovery of a main constructional feature common to all sorts of these larger flagella and cilia—how they appear always to include at least a cylinder of nine subfibrils enclosing two others. The peculiar geometry of this combination is something to grip one's attention immediately, especially the crystallographer's, so used to close-packed structures. It is beyond question a master key to the motility mechanism and I propose to talk about it again on the appropriate occasion, when I trust many of us will contribute to a discussion that should prove invaluable. We have here, a summons and stimulus to us all, as beautiful and versatile a piece of molecular biological ingenuity as may well be imagined.

The innermost, yet I believe the farthest reaching, clue is locked up for the present, however, in the detailed structure of the finer flagella, which share properties with the visibly-(9+2) flagella, as we may call them, and with muscle. From the X-ray and accessory evidence, indeed, they may be looked upon as frankly monomolecular muscles, a correlation sufficiently striking in any event; but they seem to be able to perform essentially the same kinds of rhythmic movements as the larger flagella besides. Their thickness when dry is only about 120 Å., but that must be enough for all that is really necessary.

With muscle, at the other extreme, we are bothered by 'trappings'. In a way it should be simpler—similar direct pulls instead of a system of periodically distributed pulls—and actually the problem has been brought down now to little more than the properties of actomyosin fibrils and filaments; but precisely how they operate is still, to put it colloquially, 'anybody's guess' to judge by the steady flow of ideas that come to my notice at any rate. They all amount in the end, though, to a component which must shorten at the molecular level; and I see no powerful reason yet to depart from the view that this component is the polypeptide chain and that the final effect is part and parcel of the configurational transformations of the k-m-e-f group.

Bailey and Perry will give us the biochemist's current viewpoint on the nature and organization of the components of the myofibril, prior to Hanson and Huxley's account of notable advances that have lately been made with the electron microscope—the localization, in striated muscle, of the myosin and actin constituents of the actomyosin complex, and a closer linking up with small-angle X-ray diffraction diagrams. Some of these findings also are disputed, I am afraid, while the interpretation gets 'curiouser and curiouser'; but clearly the new observations are converging

rapidly on the answer. They may be helped not a little by those just announced by Philpott and A. G. Szent-Györgyi, who have demonstrated in the electron microscope a very elegant axial periodicity of about 420 Å. in 'light meromyosin', one of the two constituents into which myosin can be split by trypsin. This matches the periodicity in the X-ray diagram of skeletal muscle that has been shown to be associated with the presence of the actin component. In the side-to-side interaction of myosin and actin, on which so much of the muscle argument turns, we expect, before ever the chemistry comes into play, at least some sort of dimensional correspondence, and here, it seems to me, we are on the track of it at last.

High up, these days, in all considerations of muscular activity we find adenosine triphosphate (ATP); its interaction with myosin, splitting off phosphate, is held to be the place where chemical energy is made available for transformation into work. And following the lead given by the Szent-Györgyi school, much attention is being focused on the properties of muscle fibres 'immobilized' by extraction with glycerine-water solvent (they can be stored in this state), and the way they contract again immediately on immersion in ATP solution. This central reaction, thus isolated as it were, will be the theme of Weber's talk, but in relation not simply to muscle but to cellular motility mechanisms in general. Weber and his collaborators have now reached out beyond the special problem of muscular contraction and seek to characterize ATP as the ubiquitous link between metabolism and motility. He will describe how the movements of many structures, besides those of muscle, can be restarted by immersion in ATP long after they have been extracted (and killed) by treatment with glycerine and water.* No need to labour the point that the Weber generalization, if only it can be substantiated, promises to open wide a splendid new window for our thoughts on biological movement.

We shall not forget, of course, our old friend keratin, which, from being a Cinderella of protein studies, suddenly managed to jump to the front and became the very prototype of biological long-range elasticity, a position it maintains to-day almost as strongly as ever—witness our calling upon Speakman from textile circles, where indeed the name of keratin is one to conjure with. We have a great deal to learn from it yet, and especially do we need to know what additional factors are involved in the transition from the synthetic polypeptides that fit in so well with the Pauling-Corey α-helix and the natural k-m-e-f family that still conforms only partially.

It is true that there is a tremendous increase in complexity of amino-acid distribution on passing from, say, poly-alanine or poly-γ-methyl glutamate

* At the meeting, there was shown a cinematograph film of these effects as seen under the microscope.

to the hair and muscle proteins, but apart from that, this whole general question of the first or early step up from the polypeptide chain level to that of visible biological fibres is a dominant and pressing one right through our subject, from one end to the other. And being concerned as it is with betwixt-and-between regions where our existing observational equipment is all too inadequate, it rather bothers us at the present stage. There are so many situations where precisely what is happening eludes us just at the critical moment. The biogenesis of cellulose fibrils is a famous example, but nearer home from our viewpoint here, collagen too, so varied in its sudden appearances from (say) connective tissues to the crossed-fibrillar laminae of the earthworm's cuticle and the ejected threads of the sea-cucumber, would be hard to beat. Recently, however, its many-sided puzzle has developed one unusually encouraging aspect: it has been shown that, from appropriate collagen solutions containing sometimes certain other constituents, aggregates can be precipitated that not only simulate the kinds of fibrils found in nature but also include several 'anomalous' forms that offer most welcome additional information, if we can co-opt it. From results such as these, which we owe primarily to Schmitt's school and now also to Randall's, it would appear possible to start out from units consisting of one or a pair of polypeptide chains and zigzag about at will among the various types of fibrils that can be built up from them; that is to say, they are all interchangeable on suitably adjusting the conditions. In one way and another, and from two complementary groups of workers, we may look forward to hearing some eminently interesting papers and discussions on the fibrogenesis of collagen.

It is perhaps hardly necessary to add that a principal incentive especially behind experiments on the reprecipitation of collagen fibrils, and the full interpretation of all the circumstances that have to be taken into account, is the hope of throwing light on the notorious close connexion between collagen metabolism and the so-called rheumatic diseases. These ancient widespread afflictions seem at last to be stimulating something like the quality and quantity of fundamental research that is so urgently required, and at the forefront of the new attack must obviously be found molecular and fine-structural studies of the kind to which we are devoting such a good fraction of our time in this symposium. We shall touch upon the pathological molecular side—collagen 'gone wrong'—with the aid of Kellgren, who is also a clinician, and with whom we ourselves at Leeds have had an opportunity of collaborating. We used X-ray diffraction, the electron microscope and biochemical analysis in a characterization of the 'fibrinoid' material in rheumatoid lesions. The degeneration of the original beautiful collagen and related fibrils is depressing to see; and, incidentally,

we never observed any improvement in this condition when the patient was treated with ACTH or cortisone. As is now recognized from a multitude of tests, these 'wonder drugs' do not bring us, after all, anywhere near the heart of the collagen diseases.

Though by a piece of benevolent 'sharp practice' we have arranged to keep back the topically exciting muscle discussion for the final session of the symposium, more logically, I suppose, we should have concluded with the cell nucleus and the apparatus of biogenesis and reproduction, the ultimate 'trend', conscious or unconscious, of all biological research when it comes to the point. The basic molecular problem of biogenesis, we believe, has to do with some subtle combination between proteins and nucleic acids, and the headquarters of the process, we are convinced, is located in the chromosomes and their accessories. The former aspect, as I have already indicated, will be glanced at in the introductory session, but later we shall naturally renew contact with the chromosomes, too, through the papers by Mazia and Gall. In such a profound field these two contributions constitute no more than tokens, or a kind of homage, I am afraid—but 'time must have a stop'. The least important part of our symposium could very easily be expanded to fill the whole, and as for biogenesis....

I repeat, if we do not make a memorable showing, it will not be through lack of a worthy topic. Shakespeare again has the gist of it:

> And take upon 's the mystery of things,
> As if we were God's spies.

THE CHEMISTRY OF SIMPLE PROTEINS

By F. SANGER
Department of Biochemistry, University of Cambridge

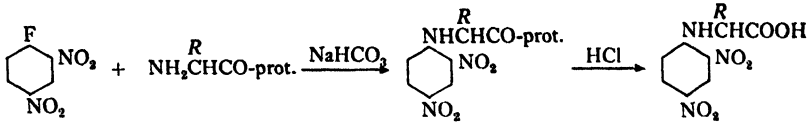
One of the major problems in protein chemistry is to determine the order in which amino-acids are laid down in protein molecules. Early investigations, notably by Fischer and by Abderhalden, met with only limited success, due to the lack of sufficiently exacting methods for fractionation of the complex mixtures produced on partial degradation of a protein. Only recently have such methods become available and these have greatly stimulated investigations into the chemical structure of proteins. The subject is thus one that is at present in an active state of development. It was reviewed in 1952 (Sanger), when attention was chiefly centred on the methods available. Since then there has been considerable progress, and this article will deal more with the results obtained and their interpretation. Two other valuable reviews by Desnuelle (1953*a*, *b*) have also appeared recently.

Two methods of approach are available for investigating the relative positions of amino-acids in protein molecules. The most general method is by the investigation of peptides produced on partial hydrolysis. From their structure it is possible to deduce sequences that were present in the original protein. In this way the complete amino-acid sequence of insulin was worked out and certain sequences have been demonstrated to be present in some more complex proteins. By this approach the exact position of any one amino-acid can only be determined when the whole sequence is known. A second method of approach locates certain amino-acids by virtue of their occupying definite positions in the protein chains. These are the terminal residues, which differ chemically from the other residues in that they have a free amino (*N*-terminal residues) or a free carboxyl group (*C*-terminal residues) and so may be identified by suitable labelling techniques. These latter methods, which have now been applied to a large number of proteins, will be considered first.

TERMINAL RESIDUES

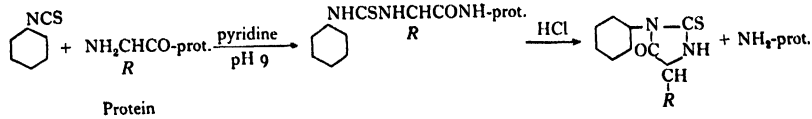
N-terminal residues

In Table 1 are listed the various *N*-terminal residues identified in proteins. Most of them have been studied by the dinitrophenyl (DNP) method (Sanger, 1945), which depends on the following reaction:



1:2:4 fluorodinitrobenzene (FDNB) | Protein | DNP-protein | DNP-amino-acid

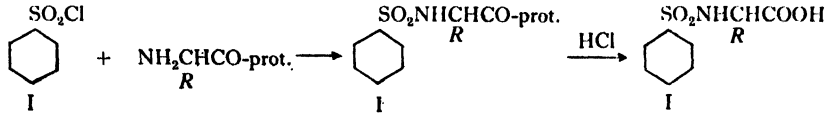
The only other method that has been used to any extent is the phenylthiocarbamyl (PTC) method of Edman (1950, 1953):



Phenylisothiocyanate | Protein | PTC-protein | Phenylthiohydantoin

This method has come into prominence during the last few years and should prove very useful, especially for the step-wise degradation of peptide chains (see below). No attempt will be made to compare the two methods, since they have not been studied to equal extents, and in many ways they are complementary. The great complexity of the structure of proteins and the large number of chemical residues present make it extremely difficult to predict exactly how any one reagent will react. Every method available will thus have certain limitations which may not be present when another method is used.

A third method which has only been used on a few proteins is the *p*-iodophenylsulphonyl method (pipsyl) (Udenfriend & Velick, 1951):



Pipsyl chloride | Protein | Pipsyl protein | Pipsyl amino-acid

Here radioactive isotopes are used, which makes possible accurate estimations of the pipsyl derivatives on a very small scale.

All these methods depend on the quantitative reaction of the reagent with the free amino groups of the protein and subsequent degradation to give substituted derivatives of the *N*-terminal residues, which can be differentiated from the remaining unsubstituted amino-acids of the rest of the protein chains, and may be estimated by suitable methods.

Since there are no synthetic proteins of known structure to use as standards, it is difficult to apply any test to these methods and to know to what extent the results obtained are correct and accurate. This can only be determined by carrying out investigations by other methods, and if satisfactory agreement is obtained, confidence may be placed in the results. Thus for insulin, the DNP method indicated the presence of one *N*-terminal phenylalanyl and one *N*-terminal glycyl residue per molecule of molecular weight 6000. On the basis of this result the molecule was split into two separate polypeptide chains, one with a phenylalanyl and one with a glycyl *N*-terminal residue (Sanger, 1949*a*). The complete sequence determination in these chains confirmed the nature of the *N*-terminal residues, and more recent results using the PTC method were also in agreement. Thus one may feel fairly certain that the *N*-terminal residues of insulin are glycine and phenylalanine. However, proteins differ greatly in their structure and complexity, so that it does not follow that results obtained with other proteins will be equally exact.

The DNP method has been studied in a number of laboratories, so that more is known about its limitations and its applicability. In considering results obtained the following factors must be considered:

(1) It is assumed that the reagent will react quantitatively with the free α-amino groups to give the DNP derivative. This is the case with free amino-acids and simple peptides. However, there is the possibility that steric and solubility factors may prevent complete reaction in the case of proteins. That complete reaction is obtained in most proteins is suggested by the fact that the amount of ϵ-DNP-lysine detected in the hydrolysate is equal to the total lysine content of the protein. In other words, the free amino groups on the lysine side-chains react quantitatively, so that by analogy one would expect the α-amino groups to do the same. Some doubt in this argument must, however, be raised by the results of Udenfriend & Velick (1951). They used the pipsyl method to determine the *N*-terminal residues of insulin and horse globulin. The results agreed qualitatively with those obtained by the DNP method, but were quantitatively considerably lower, although complete reaction with the ϵ-amino groups of lysine was obtained in both cases. Thus if incomplete reaction is obtained with this reagent, there is the possibility that the reaction is still incomplete with the DNP or PTC method. Clearly from this point of view the results should be regarded as minimum values. More confidence may be placed in them if they do not increase when more drastic reaction conditions are used or when the protein is first denatured. In certain native proteins such as β-lactoglobulin (Porter, 1948) the ϵ-amino groups of lysine do not react quantitatively but do if it is first denatured. With the DNP method no

increase in the free α-amino groups of any protein have yet been detected on denaturation. It appears probable, however, that the *C*-terminal residues of trypsin and ovalbumin are not available to carboxypeptidase in the native state (see below).

(2) If a reagent is to be used for labelling exclusively the free amino groups of proteins, the conditions of substitution must be sufficiently mild not to cause any hydrolysis of peptide bonds within the protein. The reaction with FDNB takes place at room temperature in bicarbonate solution which would hardly be expected to bring about any hydrolysis and in most cases certainly does not. However, it appears that in certain proteins where the *N*-terminal residue is aspartic acid or asparagine, the DNP amino-acid is split off during the treatment, thereby liberating a new free amino group which was on the second residue and appears as a new end-group (Weygand & Junk, 1951; Thompson, 1953). The exact reason for this unusual lability is not clear. It does not seem to be a general phenomenon and does not happen with all *N*-terminal aspartic acid residues, e.g. in serum albumin or human γ-globulin.

The reaction conditions necessary for the PTC method are somewhat more violent than those used for the DNP method, so that the possibility of hydrolytic reactions must be considered. The results of Felix (1953) with certain protamines may be significant in this connexion. Thus, for instance, according to the DNP and the pipsyl methods salmine has proline as the only *N*-terminal residue, whereas the hydantoins of both proline and valine were obtained by the PTC method. Similar results were obtained for other protamines. In the case of clupeine, proline and alanine hydantoins were obtained, whereas the DNP method indicated that alanine was the second residue.

(3) The chief source of error in using the DNP method quantitatively is due to the breakdown of the DNP amino-acids on hydrolysis of the DNP protein. Usually a correction factor is applied which is determined by treating the pure DNP amino-acid with acid in the presence of the DNP protein. It varies with the amino-acid and slightly with the nature of the protein. The presence of tryptophan in the protein appears to lead to increased destruction of the DNP derivatives (Thompson, 1951). The correction factors also appear to vary with different authors and probably depend on the nature of the acid used, the amount of light present during hydrolysis and other undefined factors. For most amino-acids the breakdown is not extensive and results to the nearest residue can usually be obtained. The only DNP derivative that is completely unstable in boiling 20% HCl is that of proline, so that although *N*-terminal proline residues can usually be detected they cannot be estimated directly. Schramm & Braunitzer

Table 1. *N-terminal residues of proteins*

Protein	Molecular weight	*N*-terminal residues	Number per molecule	Method used	Reference
Fibrous proteins					
Collagen	—	None	—	DNP	Bowes & Moss, 1951; Grassman & Hörmann, 1953
Keratin (wool)	$1{\cdot}6 \times 10^6$ (assumed)	Valine	4	DNP	Middlebrook, 1951
		Alanine	2		
		Glycine	8		
		Threonine	8		
		Serine	2		
		Glutamic acid	2		
		Aspartic acid	1		
Flagella (*Proteus vulgaris*)	10^5 (assumed)	Alanine	1	DNP	Weibull, 1953
Fibrinogen (cattle)	450,000	Tyrosine	3–4 (2)*	DNP	Bettelheim & Bailey, 1952; Lorand & Middlebrook, 1952
		Glutamic acid	2 (1)		
Fibrin (cattle)	450,000	Tyrosine	3–4 (2)		
		Glycine	4 (4)		
Fibrinopeptide A (cattle)	2,000–3,000	Glutamic acid	1	DNP	Bettelheim & Bailey, 1952
Fibrinopeptide B (cattle)	—	None	—		
Fibrinogen (human)	450,000	Tyrosine	(2)	DNP	Lorand & Middlebrook, 1953
		Alanine	(1·5)		
Fibrinogen (horse)	—	Tyrosine	—	DNP	Bailey & Rosemeyer (unpubl.)
		Threonine	—		
Muscle proteins					
Myosin	—	None	—	DNP	Bailey, 1951
Actin	—	None	—	DNP	Locker, 1954
Tropomyosin	—	None	—	DNP	Bailey, 1951
Myoglobin (horse)	17,000	Glycine	1	DNP	Porter & Sanger, 1948
				PTC	Ingram (unpubl.)
Myoglobin (whale)	17,000	Valine	1	DNP	Schmid, 1949
Protamines					
Salmine	—	Proline	1	DNP	Porter & Sanger, 1948
				PTC	Landmann *et al.* 1953*a*
				Pipsyl	Velick & Udenfriend, 1951
Clupein	—	Proline	—	DNP	Felix *et al.* 1950
Iridine	—	Proline	—	DNP	Felix & Krekels, 1953
Truttine	—	Proline	—		
Fontinine	—	Proline	—		
Sturine	—	Alanine	—		
		Glutamic acid	—		
Hormones					
Insulin: Cattle, Pig, Sheep	6,000	Phenylalanine	1	DNP	Sanger, 1945
		Glycine	1	PTC	Fraenkel-Conrat & Fraenkel-Conrat, 1951; Landmann *et al.* 1953*a*
				Pipsyl	Udenfriend & Velick, 1951
Growth hormone (cattle)	49,000	Phenylalanine	1	DNP	Reid, 1951
		Alanine	1		
Corticotrophine A	—	Serine	—	DNP	Landmann *et al.* 1953*b*
				PTC	
Glucagon	—	Histidine	—	DNP	Staub *et al.* 1954
Enzymes					
Ribonuclease	13,500	Lysine	1	DNP	Anfinsen *et al.* 1952
Aldolase (rabbit)	140,000	Proline	2	Pipsyl	Udenfriend & Velick, 1951
'Old yellow enzyme'	—	Glutamic acid	—	DNP	Weygand & Junk, 1951
		Aspartic acid			
Carboxypeptidase	34,000	Asparagine	1	DNP	Thompson, 1953
Pepsin	35,500	Leucine	1	DNP	Williamson & Passman, 1952
Trypsinogen (cattle)	20,000	Valine	1	DNP	Rovery *et al.* 1952
				PTC	Rovery *et al.* 1953*b*
Trypsin (cattle)	20,000	Isoleucine	1	DNP	Rovery *et al.* 1952
				PTC	Landman *et al.* 1953*b*; Rovery *et al.* 1953*b*

* Figures in brackets are those of Lorand & Middlebrook, the others those of Bettelheim & Bailey.

Table 1 (*cont.*)

Protein	Molecular weight	*N*-terminal residues	Number per molecule	Method used	Reference
Enzymes (*cont.*)					
Pancreatic trypsin inhibitor	9,000	Arginine	1	DNP	Green & Work, 1953
Soybean trypsin inhibitor	24,000	Aspartic acid	1	DNP	Neurath & Davie, 1954
Chymotrypsinogen (cattle)	21,500	None		DNP	Desnuelle *et al.* 1951
				PTC	Landman *et al.* 1953*b*; Rovery *et al.* 1953*b*
α-Chymotrypsin (cattle)	21,500	Isoleucine	1	DNP	Desnuelle *et al.* 1952
		Alanine	1	PTC	Landman *et al.* 1953*b*; Rovery *et al.* 1953*b*
β and γ-Chymotrypsin	21,500	Isoleucine	1	DNP	Rovery *et al.* 1953*a*
		Alanine	1		
δ-Chymotrypsin	21,500	Isoleucine	1	DNP	Rovery *et al.* 1954
Papain	20,300	Isoleucine	1	DNP	Thompson, 1954
Blood proteins					
Haemoglobin:					
Horse	66,000	Valine	6	DNP	Porter & Sanger, 1948
				Pipsyl	Udenfriend & Velick, 1951
				PTC	Ingram (unpubl.)
Donkey	66,000	Valine	6	DNP	Porter & Sanger, 1948
Cattle, Sheep, Goat	66,000	Valine	2	DNP	Porter & Sanger, 1948
		Methionine	2	DNP	Porter & Sanger, 1948
Human	66,000	Valine	5	DNP	Porter & Sanger, 1948
Foetal haemoglobin:					
Human	66,000	Valine	2–3	DNP	Porter & Sanger, 1948
Cattle	—	Valine	—	DNP	Rovery & Fabre, 1953
		Methionine	—	DNP	Rovery & Fabre, 1953
Serum albumin:					
Cattle	69,000	Aspartic acid	1	DNP	van Vunakis & Brand, 1951; Desnuelle *et al.* 1951
				PTC	Fraenkel-Conrat, 1953
Human					
Horse	69,000	Aspartic acid	1	DNP	van Vunakis & Brand, 1951; Desnuelle *et al.* 1951
Pig	69,000	Aspartic acid	1	DNP	Desnuelle *et al.* 1951
				PTC	Rovery & Desnuelle, 1954
γ-Globulin:					
Rabbit	160,000	Alanine	1	DNP	Porter, 1950
Human	160,000	Aspartic acid	1	DNP	Putnam, 1953
		Glutamic acid	1–2	DNP	McFadden & Smith, 1953
Egg proteins (hen)					
Lysozyme	14,000	Lysine	1	DNP	Green & Schroeder, 1951; Thompson, 1951
				PTC	Landmann *et al.* 1953*a*
Ovalbumin	—	None	—	DNP	Desnuelle & Casal, 1948
				PTC	Fraenkel-Conrat, 1953
Conalbumin	87,000	Alanine	1	DNP	Fraenkel-Conrat & Porter, 1952
Ovomucoid	28,000	Alanine	1	DNP	Fraenkel-Conrat & Porter, 1952
				PTC	Fraenkel-Conrat & Fraenkel-Conrat, 1951
Avidin	60,000	Alanine	3	DNP	Fraenkel-Conrat & Porter, 1952
Milk proteins					
β-Lactoglobulin	40,000	Leucine	3	DNP	Porter, 1948
				PTC	Fraenkel-Conrat & Fraenkel-Conrat, 1951
α-Casein	100,000 (assumed)	Arginine	10·7	DNP	Mellon *et al.* 1953
		Lysine	1·5	DNP	Mellon *et al.* 1953
β-Casein	100,000 (assumed)	Arginine	5·3	DNP	Mellon *et al.* 1953
		Lysine	2·4	DNP	Mellon *et al.* 1953
Other proteins					
Gliadin	27,500	Histidine	3	DNP	Köves, 1950
Tobacco mosaic virus	50×10^6	None	—	DNP	Fraenkel-Conrat & Singer, 1954
				PTC	Fraenkel-Conrat & Singer, 1954

(1953), however, have described a method for the estimation of *N*-terminal proline residues from the amount of dinitrophenol which was produced in the hydrolysate by the decomposition of DNP proline.

In this respect the PTC method is more satisfactory. The hydantoins are very stable to acid and only mild conditions are required to liberate them from the protein chains. The pipsyl derivatives are also very stable to acid, and this would appear to be the most satisfactory method for estimating proline end-groups.

In spite of the above limitations which have been emphasized, the results in Table 1 are probably mostly correct. The quantitative results have been given to the nearest residue. It is clear from these results that there is no one amino-acid or group of amino-acids which occurs predominantly in the *N*-terminal position. Almost all of the commonly occurring amino-acids have been found as *N*-terminal residues.

N-terminal sequences

Both the DNP and the PTC methods have been used for determination of *N*-terminal sequences. In the case of the DNP method the DNP protein is subjected to partial hydrolysis and DNP peptides can be identified. This method is clearly limited to relatively small peptides. At present the chief limitation is due to the insolubility of the larger DNP peptides which renders them difficult to fractionate. The largest DNP peptide that has been obtained from oxidized insulin was the octapeptide, DNP—Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala, which was fractionated by ionophoresis in aqueous dioxane (Sanger, unpublished).

In theory the PTC method is more suitable for determining *N*-terminal sequences. The PTC protein may be decomposed to the hydantoin under conditions (preferably anhydrous) which do not cause any hydrolysis of other peptide bonds. Thus the remaining peptide chain is left intact with the amino group of the second residue free. The process may then be repeated to liberate the second residue as a hydantoin. By this stepwise degradation procedure it should then be possible to work all along a protein provided there is only one type of chain present. In practice it has been possible to determine sequences up to about five residues from the *N*-terminal group. The main limitations at present are due to the reaction not being absolutely quantitative and to partial hydrolysis of peptide bonds during the degradation. It is, however, to be expected that the results will be improved upon and the development of this method should prove very profitable.

Some *N*-terminal sequences determined by these methods are recorded in Table 2.

Table 2. *N-terminal sequences of proteins*

Protein	*N*-terminal sequence	Method	Reference
Clupeine	Pro.Ala.Arg.(Thr, Ser).(Val, Ileu)	DNP	Felix, 1953
Insulin	Phe.Val.Asp(NH_2).Glu(NH_2)	DNP	Sanger, 1949*b*
	Gly.Ileu.Val.Glu.Glu	PTC	Landmann *et al.* 1953*a*
Corticotrophin A	Ser.Tyr	DNP, PTC	Landmann *et al.* 1953*b*
Ribonuclease	Lys.Glu.Thr.Ala	DNP	Anfinsen *et al.* 1954
Carboxypeptidase	Asp(NH_2).Ser	DNP	Thompson, 1953
Trypsinogen	Val.$(Asp)_{4-6}$Lys	DNP	Desnuelle & Fabre, 1954
Trypsin	Ileu.Val.Gly	DNP	
α-Chymotrypsin	Ileu.Val	DNP	Rovery & Fabre, 1953
Papain	Ileu.Pro.Glu	DNP	Thompson, 1954
Haemoglobin (horse)	Val.Leu	DNP	Sanger, 1948
	Val.Glu(NH_2).Leu		
γ-globulin (rabbit)	Ala.Leu.Val.Asp	DNP	Porter, 1950
Lysozyme	Lys.Val.Phe.Gly	DNP	Schroeder, 1952
		PTC	Landmann *et al.* 1953*a*

C-terminal residues

The study of *C*-terminal residues is less highly developed than that of *N*-terminal residues of proteins. Several methods (see Jutisz, 1954) have been proposed and applied to simple peptides, but only a few have been used with proteins.

(1) *Carboxypeptidase.* The most widely applied is the use of carboxypeptidase which specifically splits those amino-acids which have a free carboxyl group. The first amino-acid to be liberated by the action of carboxypeptidase can thus be assumed to be the *C*-terminal residue. The method is not universally applicable, since some residues such as proline and the basic amino-acids are not attacked, and in certain cases the results cannot be unequivocally interpreted, especially if more than one chain is present.

Ideally carboxypeptidase can also be used for the step-wise degradation of a peptide chain to determine the *C*-terminal sequence from the order in which the amino-acids are liberated. In most cases the results cannot be interpreted uniquely, but from careful estimations of the rate of liberation of amino-acids, Locker (1954) was able to deduce the *C*-terminal sequences of tropomyosin and actin as —Ala.Ileu.Met.Thr.Ser.Ileu and —His.Ileu.Phe respectively, and Anfinsen, Redfield, Choate, Page & Carroll (1954) demonstrated —Met.Tyr.Ala.Phe.Leu.Val as the most probable *C*-terminal sequence of ribonuclease. Three chemical methods have been proposed and applied to a limited number of proteins (see Table 3).

(2) *Reduction.* By reduction of a protein with $LiAlH_4$ or of a protein ester with $LiBH_4$, the carboxyl groups are converted to hydroxyl groups

and the amino-alcohols produced may be identified and estimated (see Chibnall & Rees, 1953; Fromageot & Jutisz, 1953).

Table 3. *C-terminal residues of proteins*

Protein	Molecular weight	*C*-terminal residue	Number per molecule	Method*	Reference
Collagen	—	None	—	Thio	Grassmann & Hörmann, 1953
Myosin	300,000	Isoleucine	1	Cbx	Locker, 1954
Actin	70,000	Phenylalanine	1	Cbx	Locker, 1954
Tropomyosin	53,000	Isoleucine	1	Hyd	Locker, 1954
Clupeine	—	Arginine	—	Acetylation	Dirr & Felix, 1931
Insulin (cattle)	6,000	Alanine	1	Cbx	Lens, 1949; Harris, 1952
		Asparagine	1	Red	Chibnall & Rees, 1953; Fromageot & Jutisz, 1953
				Thio	Waley & Watson, 1951; Turner & Schmerzler, 1954
Glucagon	—	Threonine	—	Cbx, Thio	Staub *et al.* 1954
Ribonuclease	13,500	Valine	1	Cbx	Anfinsen *et al.* 1952
Trypsinogen	20,000	None found	—	Cbx	Davie & Neurath, 1952
Trypsin	20,000	Lysine	—	Cbx	Davie & Neurath, 1952
Soybean trypsin inhibitor	24,000	Leucine	—	Cbx	Neurath & Davie, 1954
Chymotrypsinogen	21,500	None	—	Cbx	Gladner & Neurath, 1953
α-Chymotrypsin	21,500	Tyrosine	1	Cbx	Gladner & Neurath, 1953
		Leucine	1	Cbx	Gladner & Neurath, 1953
δ-Chymotrypsin	21,500	Leucine	—	Cbx	Rovery *et al.* 1954
Lysozyme	14,000	Leucine	1	Cbx	Thompson, 1952; Harris, 1952
				Red	Fromageot & Jutisz, 1953
				Hyd	Ohno, 1953
Ovalbumin		None or alanine		Cbx	Steinberg, 1953
				Thio	Turner & Schmerzler, 1954
Ovomucoid	28,000	Phenylalanine	1	Red	Penasse *et al.* 1952
				Thio	Turner & Schmerzler, 1954
Tobacco mosaic virus	50×10^6	Threonine	3,400	Cbx	Harris & Knight, 1952

* Cbx = carboxypeptidase. Red = reduction. Hyd = hydrazinolysis. Thio = thiohydantoin.

In the case of ovomucoid and lysozyme the results agreed with those obtained by other methods. With insulin, however, besides the expected amino-alcohols corresponding to the alanine and asparagine *C*-terminal residues, a varying amount of ethanolamine and traces of other amino-alcohols were found. It is probable that these are produced by the rupture or rearrangement of certain very labile bonds, in particular the Gly—Ser bond which is present (Chibnall & Rees, 1953).

(3) *Hydrazinolysis.* When a peptide chain is treated with anhydrous hydrazine, the amino-acid whose carboxyl groups were bound in peptide linkage are converted to hydrazides, whereas those with free carboxyl groups are liberated as the free amino-acids and can be separated from the hydrazides, identified and estimated (Akabori, Ohno & Narita, 1952). Theoretically it would seem rather unlikely that a reaction of this type that depends

on the non-reaction of relatively few groups would give an exact result, and in fact in the original work rather more end-groups were found for most proteins than were to be expected. However, more recently clear-cut results have been obtained with slightly modified techniques for lysozyme (Ohno, 1953) and some muscle proteins (Locker, 1954).

(4) *Thiohydantoin method.* The original method of Schlack & Kumpf (1926), which depends on the liberation of the *C*-terminal residue as a thiohydantoin when a peptide is treated with ammonium thiocyanate in acetic anhydride, has been modified for application to proteins by several authors. The conditions required are still rather violent, but in some cases reasonable results have been obtained.

Clearly none of the methods described is ideal for general use with proteins, but many of them are complementary, and with their use it should be possible to identify the *C*-terminal residues of most proteins. The results hitherto obtained are listed in Table 3. Where different methods give the same results, one may feel fairly confident that they are correct, especially if the number of *C*-terminal residues is found to be equal to the number of *N*-terminal residues as is the case with insulin, ovomucoid, lysozyme and ribonuclease.

The use of end-group methods

The study of terminal residues may be used as a test of purity and for the identification of individual proteins. From Table 1 it can be seen that corresponding proteins from a different species of animals may have different *N*-terminal residues. This is the case for haemoglobin, myoglobin, γ-globulin and fibrinogen, but no species differences were found for insulin and serum albumin.

Results with human γ-globulin (McFadden & Smith, 1953; Putnam, 1953, 1954) show that different preparations contain varying proportions of aspartic and glutamic acid *N*-terminal residues, suggesting that there are at least two distinct proteins which are present in different proportions in different preparations. Putnam has extended these studies to a number of preparations of the abnormal globulins found in patients suffering from multiple myeloma. Electrophoretic studies had indicated that the protein produced varied from patient to patient, and this was supported by the end-group studies, which also indicated that in many cases the abnormal proteins were more homogeneous than the normal γ-globulin preparations. Thus a number of myeloma globulins had a single *N*-terminal residue (aspartic acid, glutamic acid, leucine or isoleucine). Bence Jones protein, which is excreted in the urine of certain patients with this disease, was found to contain *N*-terminal aspartic acid from several individuals, whereas one preparation contained none.

The question of whether ovalbumin contains a *C*-terminal residue is still open. Using carboxypeptidase Steinberg could find no significant amount, whereas with the thiohydantoin method Turner & Schmerzler (1954) find alanine. They suggest that the *C*-terminal residue only becomes available after denaturation, which would take place when using the latter but not the former method.

Special difficulties are encountered in applying end-group methods to fibrous proteins, due largely to their insolubility. It is also difficult in many cases to know if they are homogeneous proteins. Silk fibroin was studied by the DNP method (Zahn & Wurz, 1952), and small amounts of the DNP derivatives of glycine, alanine and serine were identified. Quantitative results were not given, but they were considered by the authors to be insignificant.

Weibull (1953) found one alanine *N*-terminal residue in flagella protein on the basis of a molecular weight of 10^5. However, under certain mild conditions the protein can be disaggregated to particles of molecular weight 40,000 which suggests that cyclic chains may be present besides an open chain with alanine *N*-terminal residue.

Besides providing data on the location of particular amino-acids in protein chains, the study of end-groups gives certain information about the gross structure of a protein and also about some protein interconversions.

Protein chains. If a protein is considered to be built up simply of open polypeptide chains, then the number of chains present in the molecule is equal to the number of *N*- or *C*-terminal residues, since each such chain has one free amino and one free carboxyl group. Thus the presence of two *N*-terminal residues in insulin suggested that the molecule was composed of two chains, and this was confirmed by further detailed structural studies. Less confirmation is available for other proteins, but the fact that most of the more thoroughly investigated ones (e.g. lysozyme, ribonuclease, ovomucoid, etc.) have the same number of *N*- and *C*-terminal residues suggests that this rule is generally applicable.

As indicated in Tables 1 and 3, certain proteins (myosin, chymotrypsinogen, collagen and probably ovalbumin) appear to have no terminal residues. This suggests that they are not open polypeptide chains, but are most probably built up of one or more cyclic chains. In the case of collagen, where the molecular weight is unknown, it is possible that the chain is so long that its end-groups could not be detected by the methods used. However, this is unlikely with the other proteins of known molecular weights.

The antibiotics 'gramicidin S' and 'tyrocidine A' are known to consist of cyclic chains, and thus it is not surprising to find cyclic proteins. It is not

at present possible to determine the number of such chains in a protein or to say how many, if any, exist in a protein which also contains open chains. Only in the case of insulin, where the full amino-acid sequence is known, can we be certain that there are no cyclic chains.

Another type of chain that must be considered is the branched chain. The presence of amino groups on the lysine side chains and carboxyl groups on the aspartic and glutamic acid side-chains suggests the possibility that other peptide chains may be combined to them by peptide linkage through their *C*- or *N*-terminal residues respectively. For most proteins it is unlikely that there is any extensive branching from the ϵ-amino groups, since the DNP method indicates that they are essentially all free. Exceptions to this rule appear to be collagen (Bowes & Moss, 1951; Heyns & Königsdorf, 1953), tobacco mosaic virus protein (Fraenkel-Conrat & Singer, 1954) and sturine (Felix & Krekels, 1953). Here branching from the amino groups is a possibility, though in the case of gelatin it is most probable that the reagent does not react quantitatively, since titration data and reaction with benzene sulphonyl chloride suggests that they are all free.

If a protein contained branched chains attached to free carboxyl groups it would be expected to have more *C*-terminal than *N*-terminal residues. Most simple proteins that have been studied in detail appear to have the same number of *N*- and *C*-terminal residues, suggesting they are built up of unbranched chains. On the other hand, tobacco mosaic virus protein (Fraenkel-Conrat & Singer, 1954), the two muscle proteins, actin and tropomyosin (Locker, 1954), and possibly ovalbumin, appear to have no *N*-terminal residue but have significant amounts of *C*-terminal residues. This could be interpreted as indicating that they are cyclic chains with a branch originating from a carboxyl group of one of the side-chains. Alternatively, the amino groups could be masked in some way.

The possibility of branched chains from the γ-carboxyl groups of glutamic acid is suggested by the presence of γ-glutamyl linkages in naturally occurring substances, such as glutathione and the capsular substance of *B. anthracis*. There is also indirect evidence that glutamic acid occurs in certain proteins linked through its γ-COOH group and with its α-COOH group free (see Fromageot & Jutisz, 1953).

From Tables 1 and 3 it is evident that the number of terminal residues in a protein is usually small, suggesting, if one assumes cyclic peptides to be absent, that most proteins are built up of long polypeptide chains. The shortest chains hitherto found in a protein are those in insulin, which contain 21 and 30 residues respectively. Several other proteins, such as lysozyme, ribonuclease and haemoglobin, contain chains of 100–200 residues long, whereas γ-globulin contains over a thousand residues in its single

chain. In no pure protein have more than two different amino-acids been found in the *N*-terminal or *C*-terminal position, which makes it probable that not more than two types of chain are found in any one protein. This is not, of course, a necessary consequence, since different chains may have the same terminal residue. Thus whereas horse haemoglobin only contains valine *N*-terminal residues there are two different types of chain present which differ in their *N*-terminal sequence (Val.Leu and Val.Glu(NH_2).Leu). It should be possible, as further results are obtained, to see if this generalization is in fact true. Only in the case of wool are there more than two different amino-acids found as *N*-terminal residues, and this might be taken as evidence that wool is in fact a mixture of different proteins.

Activation of zymogens

End-group studies have given considerable information on the mechanism whereby the zymogens, trypsinogen and chymotrypsinogen, are converted to the corresponding proteolytic enzymes (see Tables 1, 2 and 3). Since these conversions are catalysed by the enzymes themselves it had been assumed that they were proteolytic, and this was supported by the following investigations.

Activation of trypsinogen. With the DNP and PTC methods both trypsinogen and trypsin were shown to contain single chains with the *N*-terminal sequences, Val.$[Asp]_{4-6}$ Lys and Ileu.Val.Gly respectively. When trypsinogen is converted to trypsin there is a liberation of a peptide which has the structure Val $[Asp]_{4-6}$ Lys (Davie & Neurath, 1952). This is clearly the *N*-terminal peptide and the conversion may be represented by the following equation:

$$\underset{\text{Trypsinogen}}{\text{Val.}\,[\text{Asp}]_{4-6}\,\text{Lys.Ileu.Val.Gly}\sim\sim\sim\sim\sim\sim} \xrightarrow{\text{Trypsin}} \underset{\text{Trypsin}}{\text{Val.}\,[\text{Asp}]_{4-6}\,\text{Lys}} + \underset{\text{Trypsin}}{\text{Ileu.Val.Gly}\sim\sim\sim\sim\sim\sim}$$

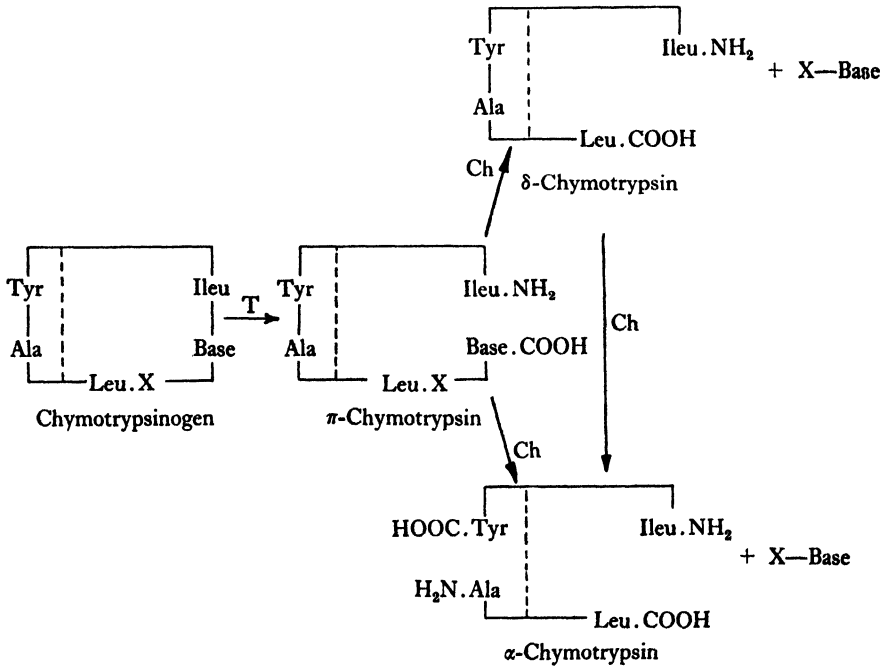
Using carboxypeptidase no *C*-terminal residue could be found in either of the native proteins, but after denaturation lysine could be liberated from trypsin.

Activation of chymotrypsinogen. Chymotrypsinogen appears to contain no *N*- or *C*-terminal residues and thus probably consists of one or more cyclic chains. When purified as the diisopropylphospho derivative α-chymotrypsin, which is the main product of the activation process, contains two *N*-terminal residues, alanine and isoleucine, indicating that two bonds are split during the reaction. Two chains are thus present, but there is only

a slight decrease in molecular weight so that they must be held together, presumably by disulphide cross-linkages.

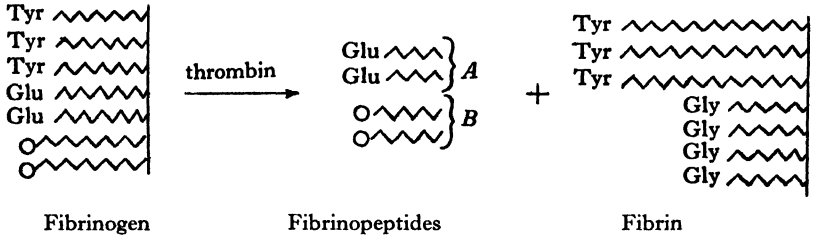
When α-chymotrypsin is treated with carboxypeptidase equivalents of tyrosine and leucine are liberated simultaneously, and these were interpreted as being the *C*-terminal residues of the two chains.

Recently Rovery, Poilroux & Desnuelle (1954) have isolated a new form of chymotrypsin (tentatively referred to as δ-chymotrypsin) which appears to be an intermediate between chymotrypsinogen and α-chymotrypsin and contains a single *N*-terminal residue (isoleucine) and a single *C*-terminal residue (leucine). The simplest explanation of these results would be that the initial activation takes place by hydrolysis of a Leu—Ileu bond giving rise to δ-chymotrypsin, and to obtain α-chymotrypsin a second bond Tyr—Ala is split. However, the initial activation of chymotrypsinogen is catalysed by trypsin, and from what is known of the specificity of trypsin, it is unlikely that it would hydrolyse a Leu—Ileu bond. Only bonds involving the carboxyl group of the basic amino-acids lysine and arginine have been found to be attacked by trypsin. The following scheme for the activation process has therefore been proposed:



This scheme involves the production of a basic peptide (X—base) during the activation process, for which Gladner & Neurath (1953) consider there is evidence.

Conversion of fibrinogen to fibrin. The fact that there was no appreciable loss of material when fibrinogen is converted to fibrin had convinced many workers that no hydrolysis was involved in this reaction, which is catalysed by thrombin. However, a study of the *N*-terminal residues of the two proteins showed that whereas fibrinogen has tryosine and glutamic acid end-groups, fibrin has tryosine and glycine. The production of new end-groups suggests that a proteolysis has occurred, and this was confirmed by the finding that peptide material (fibrinopeptide) is liberated on clotting. Two different peptides (A and B) are present. Peptide A contains glutamic acid end-groups which are presumably derived from those of fibrinogen. It contains a large amount of glycine and some serine but no alanine, tyrosine or lysine. Peptide B, on the other hand, has no detectable *N*-terminal residue and contains alanine, tyrosine and lysine but no serine. Since the number of glycine end-groups in fibrin is greater than the number of glutamic acid end-groups of fibrinogen, it was suggested that fibrinogen contains other chains with no detectable *N*-terminal residues, which give rise to free peptide B and an *N*-terminal glycine residue when treated with thrombin. The conversion may be summarized as follows:



Thrombin thus appears to be an extremely specific proteolytic enzyme. The reason why this proteolysis converts the soluble protein fibrinogen into the very insoluble fibrin is not clear but should prove an interesting field of research.

AMINO-ACID SEQUENCES IN PROTEIN CHAINS

The study of peptides produced on partial hydrolysis of proteins is largely dependent on methods for fractionation of these complex mixtures. Various efficient counter-current techniques have been developed during the last few years, of which the most useful are paper chromatography, paper ionophoresis, ion-exchange chromatography and discontinuous counter-current distribution of the Craig type. These methods work well with small peptides, but the fractionation of large peptides, such as those that are produced by enzymic hydrolysis, is somewhat less satisfactory. Ionophoresis and

counter-current distribution are the most generally applicable but do not possess the very high resolving power of the chromatographic methods.

The first peptide material to be studied was the antibiotic 'gramicidin S' (Consden, Gordon, Martin & Synge, 1947). It was subjected to acid hydrolysis and the peptides separated by paper chromatography. From their structures it was possible to deduce the unique sequence:

—Val.Ornithyl.Leu.Phe.Pro—.

Two such units are present as a cyclodecapeptide in the intact molecule.

Another similar cyclodecapeptide, tyrocidine A has recently been studied by Paladini & Craig (1954). They fractionated the peptides from an acid hydrolysate by counter-current distribution and paper chromatography and showed that the sequence was

—Phe.Phe.Asp.Glu.Tyr.Val.Ornithyl.Leu.Phe.Pro—.

It is interesting that half the molecule is identical with that of 'gramicidin S', whereas the other half is entirely different. This result may have significance from a biosynthetic standpoint, and it will be interesting to see if similar effects are found amongst proteins.

Other peptides whose structure has been determined are the hormones from the posterior lobe of the pituitary, oxytocin and vasopressin. Using both acid and enzymic hydrolysis and fractionating the peptides by ionophoresis and paper chromatography oxytocin was shown to have the structure

$$\begin{array}{c}\text{S—S}\\ \text{Cy.Tyr.Ileu.Glu}(NH_2).\text{Asp}(NH_2).\text{Cy.Pro.Leu.Gly}(NH_2)\end{array}$$

(Tuppy & Michl, 1953; du Vigneaud, Ressler & Trippett, 1953). This structure has been confirmed by synthesis; the synthetic peptide being identical with the naturally occurring hormone in both chemical and physiological properties (du Vigneaud, Ressler, Swan, Roberts, Katsoyannis & Gordon, 1953).

Although vasopressin has an entirely different biological action from oxytocin, it has a very similar chemical structure, differing only in two of the eight amino-acids.

$$\begin{array}{c}\text{S—S}\\ \text{Cy.Tyr.Phe.Glu}(HN_2).\text{Asp}(NH_2).\text{Cy.Pro.Arg.Gly}(NH_2)\end{array}$$

(Acher & Chauvet, 1953; du Vigneaud, Lawler & Popenoe, 1953).

The only protein whose amino-acid sequence has been completely determined is insulin. Its structure is shown in Fig. 1.

The molecule was initially split into the two separate polypeptide chains by oxidizing the disulphide bridges of the cystine residues to give cysteic

acid residues (Sanger, 1949*a*). Each of these chains was then partially hydrolysed by acid and the small peptides separated by ionophoresis and paper chromatography. From the structure of these peptides certain sequences could be deduced, but it was necessary to study peptides from enzymic hydrolysates before the complete structure could be determined (Sanger & Tuppy, 1951; Sanger & Thompson, 1953). In this work many more peptides were identified than were actually necessary to determine the sequence, and the fact that all of these could be explained on the basis of a unique structure added confirmation to the results and confidence that proteins could in fact be regarded as normal chemical entities.

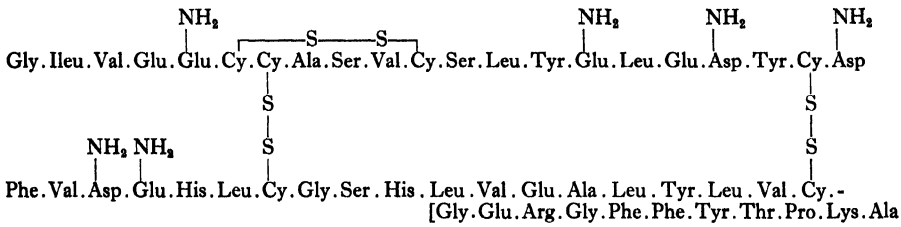


Fig. 1. The structure of insulin.

In the oxidized fractions which were investigated the cystine residues had been converted to cysteic-acid residues. In order to determine how the disulphide bonds were arranged in the insulin itself, it was necessary to obtain peptides containing cystine. This proved unexpectedly difficult since such compounds were found to be labile and to undergo an interchange reaction which led to the formation of a very complex mixture of cystine peptides, most of which had not been present in the original insulin. This reaction took place both during the normal acid hydrolysis and also during fractionation procedures in neutral or alkaline solutions. It was, however, possible to find conditions under which it could be avoided, and using ionophoretic methods of fractionation, a number of cystine peptides were identified, whose structure established the presence of the disulphide bridges shown in Fig. 1. A more detailed account of the chemistry of insulin will be published in a separate review.

All other well-characterized proteins contain considerably longer chains than those of insulin, the simplest having about 100 residues per chain. The problem of determining their sequence is thus very much more complicated, and it is uncertain whether existing methods will be adequate. Not only are the mixtures of small peptides produced by acid hydrolysis more complex to fractionate, but also to deduce a unique sequence it will be necessary to obtain a higher proportion of larger peptides, and these are harder to fractionate and in general are present in more complex mixtures than are

smaller ones. It would seem that for an initial attack on this type of protein very specific methods of degradation will be required to split the molecule into a limited number of peptides of about 10–30 residues, which could then be studied by non-specific methods of hydrolysis. For the initial specific splitting proteolytic enzymes would seem to be the most hopeful reagents. The work on insulin indicated that trypsin and chymotrypsin have a very limited specificity and would be useful, whereas other enzymes such as pepsin or papain are relatively unspecific. At present there is no very specific chemical method of degradation, though preliminary studies suggest that it may be possible to effect a specific hydrolysis at serine and threonine residues by making use of the acyl migration reaction (Desnuelle & Bonjour, 1951).

Table 4. *Peptide sequences present in lysozyme*

Sequences	References
Arg.Asp Lys.Asp Lys.Gly	Acher *et al.* 1952*a*
Arg.His.Lys Gly.Tyr Tyr.Gly	Acher *et al.* 1952*b*
Lys.Val.Phe Ser.Phe.Asp Phe.Glu	Acher *et al.* 1954
Ser.Asp.Gly.Met.Asp Thr.Asp.Val.Glu.Ala Ileu.Glu.Leu.Ala.Leu Thr.Glu.Ala Asp.Glu.Ala Leu.Thr.Ala Ala.Met.Lys.Cy.Arg Gly.Phe.Glu.Asp.Ileu Arg.Cy.Glu.Ala Thr.Pro.Gly	Thompson, A. R. (private communication)

The problem of determining the structure of other proteins has, however, already been and is being actively attacked by several workers, and a considerable number of small peptides have been identified from some of the simpler proteins.

Fromageot and his collaborators and A. R. Thompson have investigated acid hydrolysates of lysozyme, and from the peptides detected the sequences shown in Table 4 could be deduced. Other peptides have also been identified. From a tryptic hydrolysate Acher, Laurila, Thaureaux & Fromageot (1954) identified the peptide Val.Phe.Gly.Arg, which appears to be joined to the *N*-terminal sequence which thus becomes Lys.Val.Phe.Gly.Arg. The sequences shown in Table 4 account for about half of the molecule, and illustrate the value of applying these methods to other proteins.

Felix and his colleagues (see Felix, 1953) have identified a number of peptides from the protamine, clupeine, some of which are listed in Table 5. The relatively simple amino-acid composition and low molecular weight of protamines had suggested that they had a simple structure, and various formulae had been proposed based on their being composed of simple repeating units. The peptides isolated do not support any of these formulae, but they do not preclude the possibility of a somewhat less simple repeating unit. The presence of large amounts of the tetrapeptide of arginine suggests at least that the arginine residues are concentrated together in one part of the molecule.

Table 5. *Some peptides from clupeine*

(See Felix, 1953)

Arg.Arg.Arg.Arg
Ala.Arg.Arg
Ser.Arg.Arg
Ser.Ala
Val.Ala
[Val, Ala, Arg_2]
Pro.Ala.Arg (*N*-terminal)

Table 6. *Some peptides from collagen*

Peptides	References
Lys.Pro.Gly	Grassmann & Riederle, 1936
Ala.Ala.Gly Ala.Gly.Glu Gly.Asp Glu.Gly	Heyns *et al.* 1951
Thr.Gly Hypro.Gly Ser.Gly Glu.Ala	Schroeder *et al.* 1953

Another protein for which simple repeating structures have been suggested is collagen, and this has stimulated various workers (see Table 6) to study peptides from acid hydrolysates. Here again the experimental results did not support the speculative theories. In fact, studies of amino-acid sequences have in general had a destructive rather than a constructive effect on attempts to rationalize protein structure in any unified hypothesis. A study of the various sequences recorded in this article reveals no general law or principle which conditions the order or position in which amino-acids are laid down in protein chains. It would seem rather that each protein has its own unique arrangement of amino-acids, an arrangement which is conditioned not by any simple theory but rather by the limitations of the synthetic mechanism whereby it is produced and by the nature of the biological role that it has to perform.

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DEOXYPENTOSE NUCLEOPROTEINS AND THEIR PROSTHETIC GROUPS

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INTRODUCTION

Some time in 1800, the German poet Kleist wrote a letter to a friend in which he mentioned an idea that had occurred to him when passing through an arched gateway: 'Why, I thought, does the vault not collapse, though entirely without support? It stands, I replied, because all the stones want to fall down at the same time.' Similarly, one could say that the cell lives because all its components desire to die simultaneously. They survive, in other words, by getting into each other's way.

That branch of chemistry that is, among many other things, entrusted with conducting a traffic survey of the living cell is comparatively young. At the present time, it is true of cytochemistry, as essentially of many other sciences, that we can recognize the very general and the very special; but what lies between—and that is the greater part of the world—is completely obscure.

In trying to compare the state of biochemistry, for example, of fifty years ago with that of to-day, one could use a metaphor. In the old times, the knowledge of biology was perhaps similar to what could be made out in a very large, very dark house. Many objects could be more felt than seen with equal dimness, once the eyes got used to the darkness; and scientists were conscious of the limiting conditions under which they worked. In our time, however, a few very powerful and very narrow beams of light have been thrown into a few corners of this dark house, and several things can be seen in a clarity and illumination that almost distort their significance. But at the same time we have lost our dark-adaptation; and since we all have a tendency to follow the light, we have moved into these cosy corners, to the detriment of the rest, which still is, by far, the major part of nature. In pointing this out one runs the risk of being accused of trying to spread darkness.

As concerns the subject of the present conference, it will become clear that the biological significance of fibrous proteins can best be defined in those instances to which I have referred above as very special, for instance, in muscular contraction or in blood coagulation. With regard to other conjugated proteins, among which must be counted the nucleoproteins, it

is their very ubiquity that has made difficult the recognition of their function, unless one is satisfied with the trite assignment of an essentially mechanical task; but this amounts to no more than saying that these proteins are, because they are. What could, of course, be questioned is whether a purely teleological search for 'functions' is likely to prove adequate for the future development of our science. While substances and reactions endure, interpretations often perish with unseemly rapidity; and few things have as short a half-life time as the premature assignment of a function.

One more introductory remark: When speaking of nucleoproteins as fibrous proteins, one must specify that this designation refers principally to the deoxypentose nucleoproteins, though some fibrous pentose nucleoproteins are known. Furthermore, there is little indication that the carrier proteins themselves are fibrous. This character is conferred on the conjugated protein by the shape of the prosthetic group it contains, namely, the deoxypentose nucleic acids. And, strictly speaking, why these nucleic acids, or for that matter many other macromolecules of natural occurrence, exist as fibres of so marked an anisometry cannot yet be explained satisfactorily on chemical grounds.

In the following pages a brief survey of the chemistry of those nucleoproteins that can be classified as fibrous, namely, the deoxypentose nucleoproteins, will be followed by the consideration of their prosthetic groups, the deoxypentose nucleic acids. It will not be possible to do more than mention some of the current problems. For a fuller account, compare Chargaff (1955). Surveys of the chemistry of the pentose nucleoproteins have recently been published by Markham & Smith (1954) and Magasanik (1955).

DEOXYPENTOSE NUCLEOPROTEINS

Classification. The deoxynucleoproteins are usually defined as conjugated proteins in which the deoxypentose nucleic acid, functioning as a prosthetic group, is linked to the protein by electrostatic attraction or by secondary valence forces. It is, however, extremely difficult to distinguish, in macromolecules, between these two types of combination. It is, in addition, equally difficult to decide whether the nucleoprotein isolated in a given case really pre-existed in the cell or whether it was the result of a fortuitous combination of solutes present in the same extract. A genuine nucleoprotein, which in some of its properties ought to differ from a mere mixture of its components, must be regarded as a geometrically unique combination of two giant polyampholytes. It is only recently that attempts have been made to define more sharply the distinction between a nucleo-

protein and a protein nucleate artifact (compare Crampton, Lipshitz & Chargaff, 1954*a*).

Since the deoxypentose nucleic acid fraction of a given nucleus is composed of a large number of differently constituted individuals (Chargaff, Crampton & Lipshitz, 1953; Crampton *et al.* 1954*b*; Brown & Watson, 1953), a nucleoprotamine or a nucleohistone must comprise many different conjugated proteins. A search of the literature will lead to the conclusion that the number of genuine deoxypentose nucleoproteins isolated until now is small. It can, moreover, not yet be stated whether the major part, or all, of the deoxypentose nucleic acids present in the cell occurs in the form of nucleoproteins.

As regards the protein moiety, three principal groups of deoxynucleoproteins may be distinguished: (1) The nucleoprotamines occurring in ripe spermatozoa of many fish genera; (2) the nucleohistones mostly investigated in the form of calf-thymus nucleohistone; (3) the nucleoproteins in the proper sense of this term in which the nucleic acid is combined with a protein lacking the basic properties of the protamines and histones (compare Chargaff & Saidel, 1949). We are, however, very far from a satisfactory classification of the types of deoxynucleoprotein occurring in cell nuclei, especially with regard to changes in the composition of the protein moiety taking place during development and in the various phases of the mitotic cycle.

Isolation. The principal methods for the preparation of nucleoprotamines and nucleohistones, i.e. complexes in which the protein partner carries a positive charge, are based on (*a*) extraction with strong salt solutions (compare Mirsky & Pollister, 1942); (*b*) extraction with solutions of low ionic strength (compare Hammarsten, 1924). The first-mentioned procedure, widely used as the first step in the isolation of deoxypentose nucleic acids, is accompanied by an, at least partial, dissociation and may lead to artifacts. It has, on the other hand, the advantage that the activity of deoxyribonucleases is suppressed in strong salt solutions. By extraction with solutions of low ionic strength the exposure of the conjugated protein to dissociating conditions in the course of its preparation is avoided. There exists, however, the danger of a partial enzymic degradation of the nucleic acid brought about by the release of nucleases. As examples of the isolation of nucleohistone at a low electrolyte concentration the studies on calf-thymus nucleohistone by Crampton *et al.* (1954*a*) and Butler, Davison, James & Shooter (1954) may be cited; the isolation of a nucleoprotamine from sea-urchin sperm is discussed by Bernstein & Mazia (1953*a*, *b*). Preparations afforded by extraction with strong salt solutions are described, for instance, by Pollister & Mirsky (1946). The isolation from avian tubercle

bacilli of a nucleoprotein with entirely different solubility properties has been reported by Chargaff & Saidel (1949).

Properties. A real decision on the native state or the attributes of intactness of a nucleoprotein will hardly be possible before suitable biological test procedures have been found. The nucleoproteins of calf thymus and of fish sperm remain the only readily accessible deoxynucleoproteins, and most of our knowledge is derived from the study of these compounds. Nucleohistone is soluble at a very low ionic strength. As the electrolyte concentration increases, the solubility first drops rapidly, reaching a minimum in isotonic solutions, and then rises gradually. Though variations are found, freshly prepared nucleohistone is, in general, soluble in NaCl solutions stronger than 0·7 or 0·8M. Small amounts of phosphorylated impurities can be extracted from nucleohistone by 0·02–0·15M-NaCl solutions; but they usually lack the typical nucleic acid spectrum (see Table 1). The solubility properties of nucleoprotamine seem to be essentially similar.

Table 1. *Properties of calf-thymus nucleohistone solutions at different* NaCl *concentrations*

Prep. no.	Properties of supernatant fluids*	Molarity of NaCl in supernatant fluids							
		0	0·02	0·05	0·50	0·65	0·80	1·0	4·3
1	*P*	18·7	2·2	2·0	3·7	14·7	18·3	17·9	19·6
	Viscosity	1100	—	—	—	300	400	550	950
	Extinction	6250	5450	5550	5600	5500	6650	6500	6650
2	*P*	16·9	2·1	2·0	3·3	12·5	17·2	16·7	17·7
	Viscosity	1600	—	—	—	250	400	600	950
	Extinction	6450	6600	6550	6050	6200	6650	6600	6700

* The figures for *P* are in micrograms per c.c. The values for viscosity refer to $\eta_{sp.}/(P)$, i.e. the specific viscosity divided by the molarity of the solution with respect to *P*; those for extinction to $\epsilon(P)_{260}$, i.e. the atomic extinction coefficient with respect to *P*, determined at 260 mμ. (Taken from Crampton *et al.* 1954*a*.)

The absorption spectrum in the ultra-violet of a nucleoprotein is, in general, identical with that of its nucleic acid moiety (maximum around 260 mμ). No depression of the extinction coefficient of the nucleic acid component is noticeable, nor does the extent of extinction change materially under conditions of almost complete dissociation (compare Table 1). This is of interest, since the extinction of a nucleoprotein could have been expected to be less than that of the free nucleic acid, if the purines and pyrimidines of the latter were involved in the architecture of the intact conjugated protein in such a manner as to bring about the suppression of some of the chromophores (compare Magasanik & Chargaff, 1951).

The nucleic acid content of different nucleoproteins varies from about 35 to 60% of the dry weight. In most nucleohistone preparations it is around 50%. In trout and herring nucleoprotamines a phosphorus to arginine ratio of 1 has been found (Felix, Fischer, Krekels & Mohr, 1951).

Although there exists considerable evidence that deoxypentose nucleoproteins, or at any rate the nucleoprotamines and nucleohistones, occur in strong salt solutions in a largely dissociated state, in which a far-reaching separation between nucleic acid and protein has taken place, it would probably be wrong to regard such solutions as representing no more than a mixture of these components.

Dissociation and cleavage. Before the discussion of the nucleic acids proper a few words should be said about the cleavage and dissociation of the nucleoproteins, especially the nucleohistones and nucleoprotamines. The cleaving effect of high electrolyte concentrations was first observed by Bang (1904); it is of great importance for the isolation of reasonably intact deoxypentose nucleic acids.

A systematic study in our laboratory of the behaviour of nucleoproteins towards electrolytes has opened a promising approach to the problem of the fractionation of deoxypentose nucleic acids. When calf-thymus nucleohistone is exposed to increasing NaCl concentrations under conditions permitting the removal of both undissociated nucleoprotein and liberated protein, increasing quantities of sodium nucleate will be found in solution (Chargaff, 1953; Crampton *et al.* 1954*a*). Similar results are obtained when the time of contact with the salt solution is varied rather than the concentration of the latter (Lucy & Butler, 1954). When the removal of the non-dissociated portion is brought about in the presence of a denaturing phase, such as chloroform-pentanol, a large number of distinct nucleic acid fractions can be separated, as will be discussed below. The fractional dissociation of calf-thymus nucleohistone and of the nucleoprotein of wheat germ, based on previous work (Crampton *et al.* 1954*a*) and on unpublished experiments with Miss Rakoma Lipshitz, is shown in Fig. 1. These are curves taken from single experiments; but the typical shape can be reproduced easily.

Behaviour of artifacts. The behaviour of artificially prepared complexes of nucleic acids with positively charged substances has also been studied. Such nucleate compounds have been prepared not only with histone and globin (Crampton *et al.* 1954*b*), but also with synthetic polyelectrolytes, e.g. polylysine or polyvinylamine (Spitnik, Lipshitz & Chargaff, 1955). The fractional dissociation of deoxyribonucleate complexes with globin or histone is illustrated in Fig. 2. In general, globin nucleates appear to undergo dissociation more readily than histone nucleates. The relative

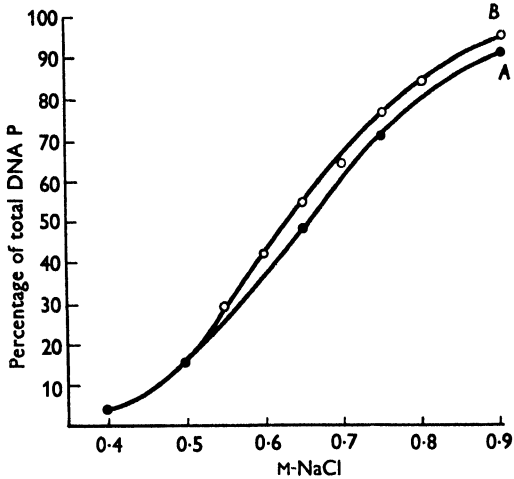

Fig. 1. Fractional dissociation of calf-thymus nucleohistone (curve *A*) and of wheat-germ nucleoprotein (curve *B*) in the presence of a $CHCl_3$ phase. The quantities of nucleic acid P (as percentage of P in the starting material) extracted at the NaCl concentrations indicated on the abscissa are plotted as the ordinate.

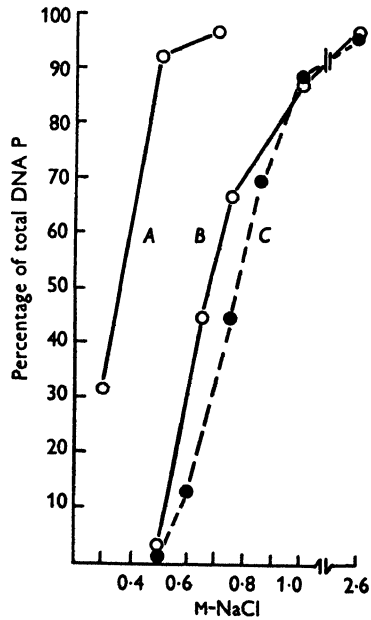

Fig. 2. Fractional dissociation, in the presence of a $CHCl_3$ phase, of artificial complexes between the deoxyribonucleic acids of calf thymus (curves *A* and *B*) and pig liver (curve *C*) with globin (curve *A*) or histone (curves *B* and *C*). Co-ordinates as in Fig. 1. (Taken from Crampton *et al.* 1954*b*.)

proportions of histone and nucleic acid and the state of polymerization of the latter are of great importance for the properties of the artifacts (Fig. 3). It can be seen that the ease of dissociation of the complexes increases with the relative amount of nucleic acid present and decreases very markedly with the viscosity of the nucleic acid. It may be mentioned that under appropriate conditions nucleohistone is rendered soluble at a low ionic strength by the addition of large amounts of degraded deoxyribonucleic acid (Crampton & Chargaff, 1955). The cationic species used also influences

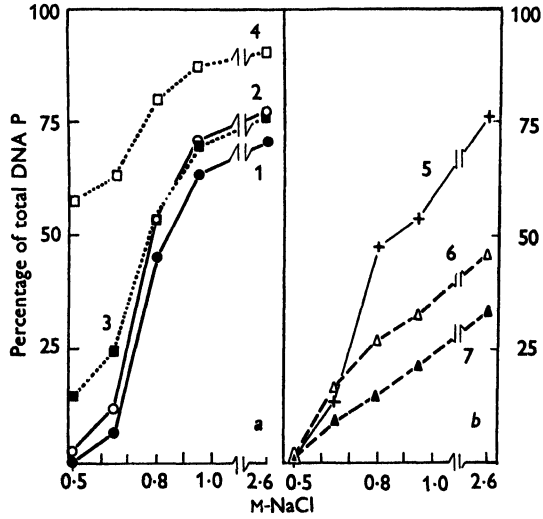

Fig. 3. Successive extraction with NaCl solutions, in the presence of a $CHCl_3$ phase, of artificial histone complexes with calf-thymus deoxyribonucleic acid. In (*a*) the amount of histone varied, in (*b*) the degree of polymerization of the nucleic acid. The weight ratio of histone to nucleic acid P was 23 (curve 1), 18 (curve 2), 14 (curve 3), 4·5 (curve 4), 17 (curves 5–7). The viscosities, as $\eta_{sp.}/(P)$, i.e. the specific viscosity divided by the molarity of the solution with respect to P, were: curve 5, 1000 (intact preparation); curve 6, 420 (0·1 % solution heated at 75° for 5 min.); curve 7, 240 (0·1 % solution heated at 75° for 40 min.). Co-ordinates as in Fig. 1. (Taken from Crampton *et al.* 1954*b*.)

the progress of the dissociation. The remarkable effect of an admixture of as little as 0·06 M-$MgCl_2$ to the NaCl solutions is shown in Fig. 4. When solutions of $MgCl_2$ alone are employed, the liberation of nucleic acid from its complex with histone occurs at even lower salt concentrations, as much as 70% of the nucleic acid P being detached by 0·3 M-$MgCl_2$ and almost the entire amount by a 0·6 M solution.

In these experiments the histone nucleate complex was formed at the lowest NaCl concentration employed for fractionation. The conditions under which the complex is formed are, however, not without influence on its subsequent behaviour. This is brought out by the experiments assembled in Fig. 5. Two differently treated nucleohistone preparations are compared

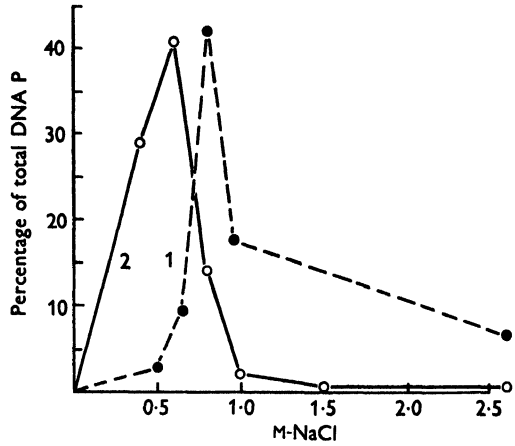

Fig. 4. Effect of magnesium ions on the fractional dissociation, in the presence of a $CHCl_3$ phase, of artificial histone complexes with calf-thymus deoxyribonucleic acid. The weight ratio of histone to nucleic acid P was 17. The successive extractions were performed with NaCl solutions of the indicated molarity in the absence (curve 1) and in the presence of 0·06 M-$MgCl_2$ (curve 2). Co-ordinates as in Fig. 1. (Taken from Crampton *et al.* 1954*b*.)

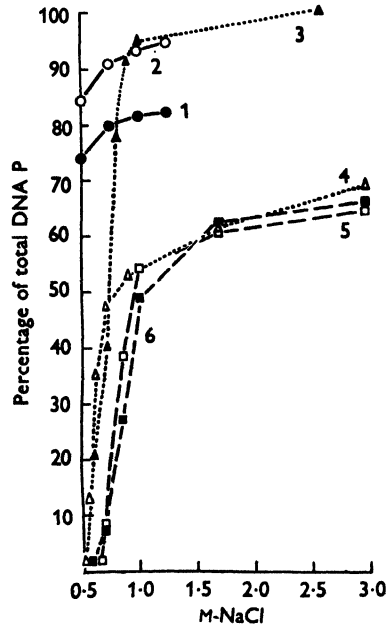

Fig. 5. Successive extraction with NaCl solutions of increasing strength, in the absence (curves 1–4) and in the presence (curves 5 and 6) of a $CHCl_3$ phase, of calf-thymus nucleohistone (curves 1 and 2) or of artificial histone complexes (curves 3–6) with calf-thymus deoxyribonucleic acid. Compare the text for experimental details. Co-ordinates as in Fig. 1. (Taken from unpublished experiments with Miss Rakoma Lipshitz.)

in curves 1 and 2. Before being precipitated by dialysis against 0·15 M-NaCl, one portion was in contact with 3 M-NaCl (curve 2), the other portion remained in distilled water (curve 1). The precipitates then were extracted successively with salt solutions of increasing strength in the absence of a denaturing chloroform phase. Similarly, artificial histone nucleates were formed in the absence of salt (curve 3) and in 3 M-NaCl (curve 4) and extracted successively. In another series, similarly produced complexes (curve 5 without, curve 6 with 3 M-NaCl) were extracted stepwise in the presence of $CHCl_3$-pentanol. These experiments permit a comparison of the behaviour of nucleohistone and of histone nucleate complexes, prepared at a low electrolyte concentration (curves 1 and 3), towards extraction with salt solutions in the absence of chloroform with that of preparations that had initially been in contact with a 3 M-NaCl solution (curves 2 and 4). The effect of chloroform on the rate of appearance of nucleic acid P in the extracts is demonstrated by a comparison of curve 3 with 6 and of curve 4 with 5. Certain characteristic differences between nucleohistone and histone nucleate are brought out when curve 1 is compared with 3 and curve 2 with 4.

Isolation of deoxypentose nucleic acids. The first step in the preparation of a deoxypentose nucleic acid is, irrespective of whether this is done consciously or not, the isolation of a nucleoprotein. Once the nucleoprotein or a mixture of nucleic acid and protein has been extracted, the removal of the protein is carried out by treatment with chloroform, saturation with sodium chloride, or with the aid of an anionic detergent. The several methods have been reviewed critically in a recent survey (Chargaff, 1955).

DEOXYPENTOSE NUCLEIC ACIDS

One of the surest signs that one has reached a stage of truth is that its affirmation begins to bore the audience. I shall, therefore, limit myself to a brief consideration of the principal conclusions regarding the composition and structure of nucleic acids, many of which already had been formulated six or seven years ago (Chargaff, 1950), i.e. in what were the early days of modern nucleic acid research.

Is there more than one deoxypentose nucleic acid in nature? All evidence points to the existence of a very large number of differently constituted nucleic acids; a number probably exceeding by far what can be revealed by constituent analysis. For it should be remembered that, while differences in nucleotide composition signify divergences in nucleotide sequence, an identical composition does not prove an identical sequence. An early estimate of the possible number of isomers having the composition of

calf-thymus deoxyribonucleic acid, which assumed a chain of 2500 nucleotides, gave the figure of 10^{1500}. Although the differentiation between nucleic acids on the basis of differences in the contents of their nitrogenous constituents is a relatively crude expedient, since it does not permit a distinction between sequence variations that are not accompanied by a change in total composition, it can be shown in many cases that nucleic acids of different origin differ in composition (compare the survey by Chargaff, 1955). To mention only the most extreme examples: the ratio of the sum of adenine and thymine to that of guanine and cytosine is about 1·9 in the deoxyribonucleic acid of *Echinocardium cordatum*, but about 0·4 in that of avian tubercle bacilli. It is, in fact, possible to make a gross distinction between three types of deoxypentose nucleic acid: (*a*) the AT type, in which adenine and thymine are the major constituents; (*b*) an intermediate type, in which all bases are present in nearly equimolar proportions (found only in *Escherichia coli*); and (*c*) the GC type, in which guanine and cytosine predominate. While, therefore, the question whether there exists more than one deoxypentose nucleic acid must be answered in the affirmative, a decision whether different tissues of the same host yield entirely identical nucleic acid specimens is more difficult. For the moment, it would appear that differences in the composition of preparations from different organs, if they exist at all, are of very doubtful significance.

Do there exist recognizable repeating units? The tetranucleotide hypothesis is incorrect. One must conclude that there is no subunit of recognizably recurrent structure larger than a mononucleotide. If repeating polynucleotide units occur in the nucleic acid chain, we have no way as yet of demonstrating their existence. The nucleic acids must be regarded as complicated and intricate high polymers of undetermined and largely arrhythmic nucleotide sequence.

Regularities. The mistakes of the present are made by trying to avoid the mistakes of the past. Though the danger of the premature imposition of regularities is well exemplified by the tetranucleotide hypothesis, it would be an error not to draw generalizing conclusions from experimentally established facts. The apodictic manner of the following enumeration is chosen to conserve space. Despite wide divergences in their nucleotide composition and sequence all deoxyribonucleic acids appear to be characterized by the following regularities (Chargaff, 1950, 1951, 1955): (*a*) The sum of the purine nucleotides equals that of the pyrimidine nucleotides. (*b*) The molar ratio of adenine to thymine equals 1. (*c*) The molar ratio of guanine to cytosine (+methylcytosine) equals 1. (*d*) The number of 6-amino groups (adenine + cytosine + methylcytosine) equals that of 6-keto groups (guanine + thymine). The last-mentioned regularity, and in some

cases also the others, but with uracil taking the place of thymine, applies also to certain pentose nucleoproteins (Elson & Chargaff, 1954).

It is, in fact, because of these relationships that it is possible to state that, for instance, in the deoxypentose nucleic acids of wheat germ 5-methylcytosine substitutes for a part of the cytosine (Wyatt, 1951; Chargaff, 1951) or that in certain bacteriophage nucleic acids 5-hydroxymethylcytosine replaces cytosine entirely (Wyatt & Cohen, 1953). It is, incidentally, remarkable that so far no purine satellites have been discovered.

But nature is inexorably approximate; and one could suspect that occasional deviations from fit are more significant than the fit itself. This may still turn out to be true of such giant molecules as the nucleic acids, but I do not believe it will. In any event, I should like to cite here a recent survey in which the results of all published analyses, carried out in several laboratories, of deoxypentose nucleic acid specimens from about fifty different genera were tabulated (Chargaff, 1955). The average values of the molar relationships that bring out the complementariness principles mentioned before were as follows.

Adenine to thymine	1·009
Guanine to cytosine (or its analogues)	1·001
Purines to pyrimidines	1·000
6-Amino to 6-keto groups	1·008

If it is considered that the nucleic acids varied very widely in their nucleotide composition (ratios of adenine + thymine to guanine + cytosine between 2 and 0·4), the agreement of the 1:1 relationships is most impressive, especially in view of the diversity of sources, preparations and procedures. No authentic case of an appreciable deviation has yet come to my attention.

Molecular structure. Deoxypentose nucleic acids are polynucleotide chains that are held together by phosphoric acid diester bridges between the deoxypentose molecules. Our knowledge of the sugar found in different preparations rests mostly on chromatographic evidence which has up to now yielded no indication of the presence of any but 2-deoxyribose (Chargaff, 1955). The position of the phosphate bridges has been studied in some detail only in the deoxyribonucleic acid of calf thymus in which it appears to be 3′:5′ (Brown & Todd, 1955). No determinations of end-groups have as yet been possible nor has the existence of a terminal phosphate group been demonstrated. Strictly speaking, it cannot yet be asserted that the deoxypentose nucleic acids occur as open and not as closed chains. It is, however, probably too early to worry about the tail before we can describe the animal.

The excellent X-ray photographs obtained at King's College (Wilkins, Stokes & Wilson, 1953; Franklin & Gosling, 1953) and the discovery of

the general unity relationships mentioned before have formed the basis of an interesting structural proposal (Watson & Crick, 1953; Crick & Watson, 1954). This model, which in the recent past has been unveiled repeatedly and sometimes very amusingly,* assumes a helical dyad in which two coiled strands are held together by a specific pairing of the nitrogenous constituents showing the unity relationships mentioned before. This conception has several attractive features; it makes good use of some of the experimentally established facts and is, on the whole, not incompatible with the X-ray (Wilkins, Seeds, Stokes & Wilson, 1953) and part of the chemical evidence. But between what is plausible and what is true in the natural sciences there lies an ocean which has seldom been crossed.

The invention of models is an essentially sterile undertaking unless two things are thereby accomplished: (*a*) a satisfying explanation of all known and sometimes seemingly contradictory facts; (*b*) an impulse to further and crucial experimentation. Therefore, before this structural hypothesis is elevated to the dignity of a natural law, a few remarks may be in order. The model provides a satisfactory explanation, though it probably is not the only possible one, of the unity relationships observed experimentally. Whether, in its detailed form, it does more than describe the structure of that portion of the processed nucleic acid preparation from which the diffraction patterns were obtained, remains, however, to be established. More work on genuine nucleoproteins, especially on compounds with proteins other than histone or protamine, e.g. of the type found in tubercle bacilli (Chargaff & Saidel, 1949), is urgently required. The existence of two discrete and complementary chains has not been demonstrated; no deoxypentose nucleic acid preparation has shown noticeable deviations from the 1:1 ratios, not even for the ratio of purines to pyrimidines.

It is, however, the second part of the hypothesis, namely, the suggestion of a copying mechanism for the mutual reproduction of the two half-chains, that is more difficult to reconcile with some of what is known. It is assumed that a specific pairing is brought about by hydrogen-bond formation between the nitrogenous bases of the two chains in such a manner that a base having a keto group at the 6 position (guanine, thymine) can bond only with a base carrying a 6-amino group (adenine, cytosine), and that a purine always bonds with a pyrimidine, thus maintaining a constant distance between the two chains. It will be observed that the model is quite strict as regards certain restrictions governing the size of the partners (purine is linked to pyrimidine) and the position of the hydrogen bonds

* 'This pairing is likely to be so fundamental for biology that I cannot help wondering whether some day an enthusiastic scientist will christen his newborn twins Adenine and Thymine!' (Crick, 1954). No doubt, this too will happen; but is 'enthusiastic' the correct epithet?

(purine-1 to pyrimidine-1; purine-6 to pyrimidine-6), but does not require the absence or presence of substituents in other positions. Thus, 5-methylcytosine or 5-hydroxymethylcytosine should be able to take the place of cytosine, and uracil and 5-methyluracil (thymine) should be similarly exchangeable, both in their positions on the 'positive' or 'negative' or during the automatic replication of the chains. That the latter replacement does not occur despite the availability of uracil is amply demonstrated by the absence of uracil from deoxypentose nucleic acids.

As regards the 5-substituted cytosine derivatives, the situation is even more disturbing. One must surely assume that both cytosine and 5-methylcytosine are available to the cell during the formation of certain deoxypentose nucleic acids, since both pyrimidines are incorporated in what appears to be constant proportions. For instance, three different groups of workers, analysing several independently prepared specimens of the deoxypentose nucleic acid of wheat germ, found the following figures for the 5-methylcytosine content (as moles per 100 g atoms of phosphorus): 5·8, 6·0, 5·7 (Wyatt, 1951; Brawerman & Chargaff, 1951; Laland, Overend & Webb, 1952). Similarly, the corresponding proportion of 5-methylcytosine in the nucleates from bovine tissues is around 1·3 (see the survey given by Chargaff, 1955). This constancy of incorporation must be due either to a specific selection operating during the reproduction of the nucleic acids or, much less likely, to an unexpectedly steady level in the supply of these pyrimidines or their precursors in the cell. In the latter case, the degree of incorporation of a pyrimidine and its 5-substituted analogue would be governed by the laws of probability and there could be no preferential accumulation of the satellite in certain portions of the polynucleotide chain or in one or the other of the fractions into which a total deoxypentose nucleic acid preparation can be separated. But the available evidence suggests that 5-methylcytosine does not replace cytosine at random and that the scheme is either incorrect or incomplete in some essential features. This evidence is twofold.

(i) During the fractionation of calf-thymus deoxyribonucleic acid, which will be mentioned in the concluding section of this article, very significant divergences in the concentration of 5-methylcytosine in the several fractions were found (compare Table 2 which is taken from the results of Chargaff *et al.* 1953). Similar results, soon to be published, have been obtained with fractions of the deoxypentose nucleic acid of wheat germ (Lipshitz & Chargaff, 1955).

(ii) The action of pancreatic deoxyribonuclease on deoxyribonucleic acids results in the production of a considerable proportion of dinucleotides which have recently been separated and analysed (Sinsheimer, 1954).

Unless the assumption is made that this enzyme distinguishes specifically between cytosine and its 5-methyl derivative, it is significant that the proportions of the various dinucleotides are far from what randomness of incorporation would predict. For instance, the frequency of methylcytosine being linked to guanine in a dinucleotide as compared with that of cytosine being so attached is 20 times as high in calf-thymus nucleic acid, and 13 times as high in the wheat-germ preparation, than would have been expected for non-selective incorporation. The unexpectedly high proportion of 5-methyldeoxycytidylic acid that is linked to deoxyguanylic acid (Smith & Markham, 1952; Sinsheimer 1954) makes one, in fact, wonder whether it is not the adjoining nucleotide rather than the one opposite that directs the incorporation.

Table 2. *5-Methylcytosine distribution in fractions of calf-thymus sodium deoxyribonucleate**

	Fraction			
	1	2	3	4
NaCl molarity in extraction of nucleohistone gel	0·40	0·45	1·7	2·6
Corrected mean proportions:				
Thymine	24·2	24·0	29·0	28·7
Cytosine	23·9	23·4	19·8	21·3
5-Methylcytosine	1·9	2·7	1·2	0
Molar ratio:				
Thymine to 5-methylcytosine	12·7	8·9	24·2	—
Cytosine to 5-methylcytosine	12·6	8·7	16·5	—

* The mean proportions of each constituent have been corrected on the assumption that one-half of the nucleic acid P is contributed by the pyrimidine nucleotides. In one hydrolysis experiment with fraction 4 a minute amount of 5-methylcytosine (about 0·6 mole) was found.

Two other findings could also be cited, though their detailed discussion would take us too far, namely, (*a*) the incorporation, from the medium, of 5-bromouracil or 5-iodouracil in the deoxypentose nucleic acids of certain deficient bacteria or of bacteriophages in replacement of part of the thymine (Dunn & Smith, 1954; Zamenhof & Griboff, 1954); and (*b*) the complete replacement of cytosine by 5-hydroxymethylcytosine in certain phage nucleic acids (Wyatt & Cohen, 1953). The latter synthesis, at any rate, may be presumed to occur in spite of the presence of the analogous pyrimidine, cytosine.

Is there more than one deoxypentose nucleic acid in a cell? An answer to this question appeared very difficult for a long time. On the one hand, many different nucleic acid preparations from the same genus showed a remarkable constancy of composition. On the other hand, it appeared rather unlikely on general grounds that a cell, which is able to produce so many different

proteins, should give rise to no more than one deoxypentose nucleic acid. But the task of separating a mixture of closely related polyampholytes of a very high molecular weight appeared hopeless; and one could ask whether a nucleic acid preparation comprising a very large number of individuals should not be treated as one compound. This is, indeed, a problem that leads directly into the epistemology of chemical identity; and many have found that it is an ungrateful task to spend one's life at the frontier of knowledge without the proper passport.

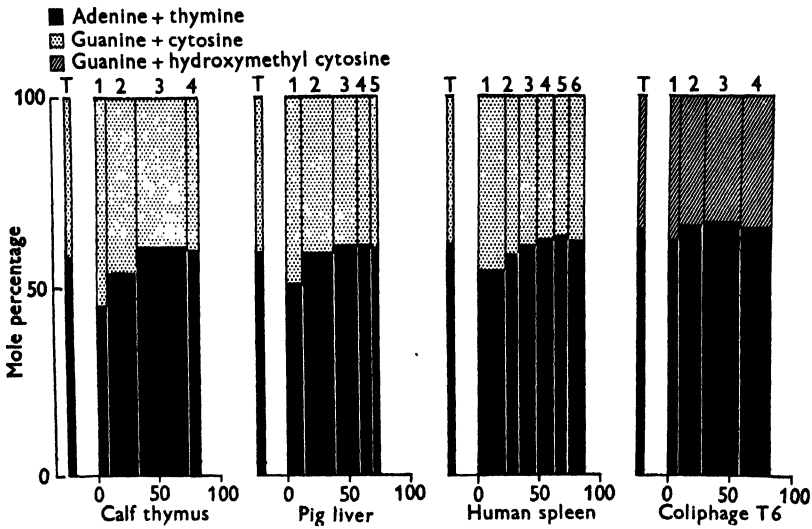

Fig. 6. Composition of fractions (in moles per 100 g. atoms of phosphorus) prepared by the fractional dissociation of artificial histone complexes with the indicated sodium deoxypentose nucleate preparations. Each block represents a fractionation run; the consecutively numbered bars within each block represent individual fractions, with the NaCl concentration of the solutions employed for extraction rising from left to right. The first column (*T*) indicates the average composition of the total deoxypentose nucleic acid preparations. The abscissa indicates the proportion of original nucleic acid P recovered in each fraction. (Based on the results of Crampton *et al.* 1954*b*; taken from Chargaff, 1955.)

Later, a process for the fractionation of deoxypentose nucleic acids was discovered which is based on the fractional dissociation of a nucleoprotein, as discussed above (see also Fig. 1). The procedure, which led to the separation of many deoxypentose nucleate preparations into a series of fractions of divergent purine and pyrimidine contents, was first applied to calf-thymus nucleohistone (Chargaff *et al.* 1953) and later extended to the fractionation of other nucleic acids through their complexes with histone, globin (Crampton *et al.* 1954*b*) or polylysine (Spitnik *et al.* 1955). Subsequently, a similar arrangement was described in a preliminary form in which the fractionation was carried out by means of a histone-kieselguhr column (Brown & Watson, 1953).

The gradual extraction of nucleohistone or of an artificially prepared protein nucleate with salt solutions of increasing strength in the presence of a denaturing phase, such as chloroform-pentanol, was found to yield a series of nucleic acid fractions with diminishing concentrations of guanine and cytosine and rising concentrations of adenine and thymine. What should, however, be stressed is that in all fractions the equimolarity of each pair of constituents and of total purines and pyrimidines is fully maintained. A selection of such fractionation experiments is shown in Fig. 6.

These findings, fully discussed in the publications cited above, suggest that the deoxypentose nucleic acid of a given cell is composed of a very large number of differently constituted individuals and that it is possible to achieve the resolution of this entire spectrum of structural gradations into several distinct 'bands', in which all the regularities characteristic of the entire nucleic acid are maintained.

There is little doubt that these fractions should not be considered as individuals. If we are dealing with a continuous spectrum, as I have suggested, it may, in fact, be impossible to isolate what could be considered as a rigorously homogeneous specimen; it is not easy even to imagine a proper criterion for such a decision. Conceivably, no two nucleic acid molecules, within the same nucleus, are entirely identical. But here we clash against the definition of a macromolecule; and seeking refuge in statistics, the science of reconciling the parallels, we may conclude that it is hard to fight on a front where friend and enemy wear the same uniform.

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THE DISTRIBUTION OF COLLAGEN AND CHITIN

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There are two principal skeletal systems which support the cellular structure of animal tissues, and these may be described as the collagenous and the chitinous skeleton. The collagenous skeleton consists principally of the unique fibrous protein, collagen, together with varying quantities of mucoid, notably chondroitin sulphate, while the chitinous system consists of the polysaccharide, chitin (polyacetylglucosamine) together with proteins which are not collagen-like; but a reasonably complete picture of the chemistry of the development process, or of the final products, is not available for either system. Both collagen and chitin are usually formed as extracellular secretions that are conspicuously fibrous and often sites for heavy deposits of calcium salts; the collagenous system is almost entirely of mesodermal origin and thus internal within the body, while the chitinous system is mainly of ectodermal origin and forms the characteristic exoskeletons. In a given animal the collagenous system would be the total of bone, tendons and connective tissue, while the chitinous system would include the hardened exoskeleton, apophyses and apodemes, the cuticle lining internal organs and the tracheal tubes.

The two systems are normally of quite different origin and could be considered as entirely independent. However, in annelids and nematodes the ectodermal cuticular system is of the collagenous type, whereas in arthropods it is chitinous. Thus the collagen and chitin systems replace one another in such supporting structures as cuticles (Reed & Rudall, 1948; Rudall, 1950). In order to gain a further insight into the possible interaction of the two systems a study was made of the distribution of collagen and chitin in a wide range of animal tissues.

The aim is not to explore the phylogeny of collagen and chitin but rather to know whether, as the chitinous system becomes very extensive, the collagen system is reduced. The quantitative estimation of chitin could be very accurate, as chitin can be isolated from most substances by treatment in hot dilute alkali. Estimation of collagen quantitatively is much less easy as insufficient is known about all different kinds of collagen. However, in the present study collagen is not being measured quantitatively, but only assessed as not detectable, detectable in small quantities, or very abundant.

We are using the wide-angle X-ray diffraction pattern to determine the presence of collagen, for it is this pattern which clearly groups together a variety of proteins whose relationship might be most difficult to establish in other ways (Astbury, 1940). By virtue of their similar diffraction patterns we assume that these proteins have something very important in common, most probably a high glycine and hydroxyproline content. If we had full confidence that the presence of hydroxyproline always meant the existence of true collagen-type structures, then the measurement of this amino-acid might give us the best estimate of the quantity of collagen. However, we are only concerned just now with estimating collagen as a structure with a characteristic wide-angle diffraction pattern (Marks, Bear & Blake, 1949).

The presence of chitin can be detected fairly reliably by various chemical tests—notably the chitosan test—and recently a detailed account of the occurrence of chitin in animals and plants has been given by Richards (1951). In the present paper chitins are considered in the light of their diffraction patterns, and differences in the patterns may be illuminating where colour tests for chitin have not given reliable differences. We have established elsewhere that chitin in natural structures rarely gives the diffraction pattern of pure chitin (Fraenkel & Rudall, 1947). To compare chitins from different sources it is necessary to bring them all to a pure or fairly pure state, and this is achieved by boiling the structure in 5 % KOH solution for 24 hr. followed by prolonged washing in cold water. The product is not chemically pure chitin but nevertheless sufficiently pure to yield diffraction patterns of highly crystalline pure chitin. Many dozens of samples of various arthropod cuticles have been purified in this way, and as tested by X-rays always gave the sharp diffraction pattern of pure chitin. If the pure chitin condition is not produced as a result of the above treatment, then it is regarded as a structure requiring more detailed analysis before it can be defined, possibly even analysis of the individual sugars.

In the study of collagen and chitin in the various types of animals as described below, the above X-ray diffraction criteria were used in defining the two kinds of structure.

COELENTERATA

The first demonstration of collagen in the simpler Metazoa was given by Astbury & Bell's study of 'jellyfish'. Undoubtedly, by this they meant one of the large scyphozoan medusae found around British coasts. The discovery was so briefly reported in the first place (Astbury & Bell, 1939; Astbury, 1940) that it led to the belief that the collagen was secreted as a free filament by the animal (see Marks *et al.* 1949). In fact the patterns were obtained

from pieces of the animal body that were dried under slight tension. These pieces were either the superficial 'skin' of the medusa or blocks of typical jelly which remained when the more coherent 'skin' was removed. The relative quantity of collagen is so great that no non-collagen material was prominent in the diffraction patterns of either the firm 'skin' or the internal jelly. One of the diffraction patterns is illustrated in Fig. 3 of Astbury's Harvey Lecture (1950). The pattern certainly arises from the mesogloea fibres—both those microscopically visible and the smaller submicroscopic units which must make up the bulk of the mesogloea gel. From these observations we should expect the mesogloea of all coelenterates to be constructed mainly of collagen material, and where these tissues have been examined by X-rays abundant collagen has been found (Champetier & Fauré-Fremiet, 1942; Marks *et al.* 1949). We thus have collagen in the two types of structure, in the characteristic jelly of the medusoid form and in the fibrous connective tissue of various anthozoans. My own observations confirm the abundant presence of collagen in the decalcified body wall and septae of *Alcyonium.*

In our present study we are particularly interested in those types which show the least development of a mesogloea. Such is the situation in hydrozoan polyps as distinct from the hydrozoan medusae where the mesogloea is well developed. In the polyps, Hyman (1940) prefers to describe that very thin structureless layer separating epidermis and gasterodermis as a 'mesolamella' indicating its possible real distinction from the mesogloea of other types. Recently Holmes (1950) viewed the epithelial muscle processes of *Clorohydra* as mesogloea fibres, and the epithelial cytoplasm, muscle process and mesogloea as one continuous protoplasmic entity. But in terms of molecular structure this does not tell us whether the mesolamella of hydrozoan polyps is of the same kind as the obvious collagenous mesogloea of scyphozoans and anthozoans or as the suspected collagen of hydrozoan medusae (Chapman, 1953). I examined the whole body of freeze-dried hydra specimens by X-rays but could detect no collagen, and would deduce that collagen is either absent or at most minutely present in hydrozoan polyps. The point then for future studies to decide is whether the mesolamella in Hyman's sense is entirely non-collagenous or what relation it bears to typical mesogloea.

The distribution of chitin in various coelenterate types is of special interest. The periderm of hydrozoans has long been described as chitinous, while the pneumatophores of Siphonophora possess a skeleton of a chitinous nature. By X-ray means I have observed that the decalcified coenosteum of millipores is likewise chitinous. Chitin has not been reported, and is probably absent in hydrozoan medusae, in scyphozoans and anthozoans.

The coenosteum of a typical madrepore upon decalcification yielded a negligible stable organic matrix and gave no evidence for a chitinous structure. The mesogloea of madrepores is described as very thin but composed of cells and fibres (Hyman, 1940).

The three coelenterate chitins studied were from *Tubularia*, *Velella* and a millipore coral. The millipore was decalcified in 5% trichloracetic acid, and the abundant coenosarc so isolated was then purified in boiling 5% KOH for 24 hr. Whole colonies of *Tubularia* and a *Velella* pneumatophore were purified in the hot alkali without previous decalcifying treatment. None of the specimens gave the highly crystalline chitin diffraction pattern that is always obtained from purified arthropod chitin. Apart from the absence of most of the detail of the true chitin pattern the 002 reflexions vary between 10 and 10·3 Å. as compared with the well-defined 002 of 9·6 Å. in pure insect chitin. Of the three samples the *Tubularia* chitin approached the standard arthropod chitin more nearly. In the case of the millipore two non-chitin diffractions were strongly present, namely, an equatorial reflexion of 6·1 Å. and strong non-axial reflexions of spacing 3·92 Å.; these seem likely to arise from a second principal constituent. At present the non-typical features of these coelenterate chitins cannot be explained. An obvious extension would be to dissolve the products in formic acid and attempt to re-precipitate the true crystalline chitin fraction, as has been done for some molluscan chitins described below. This would be a first step in the process of gaining purer products before examining the possible existence within the polysaccharide chains of sugar residues other than *N*-acetylglucosamine.

In the hydrozoan polyp the epidermis must secrete the chitinous periderm and one possibility is that it cannot secrete at the same time a collagenous mesogloea. The gasterodermis might be able to do so, though there may be a distinction between these two layers in that the inner layer does not normally contribute to mesogloea structure. Another view is that owing to the external secretion of a chitin structure the mesolamella is altered in some way that does not favour the formation of collagen-type molecules; presumably collagen and chitin molecules are only formed in an appropriate medium. In general, then, where chitin is produced in coelenterates there is apparent scarcity or even absence of collagen, while where chitin is absent collagen is most abundantly present. A few exceptions to this may occur, notably, of course, the hydras, which do not appear to produce a periderm; but *Protohydra*, for example, produces a delicate periderm and it is a question as to whether the whole mechanism of periderm formation is completely eliminated in naked forms.

PORIFERA AND ECHINODERMATA

As a result of the study by Marks *et al.* (1949) we know that the mesogloeal protein of some sponges, Keratosa, is a typical collagen and might well expect this to be the molecular plan of the mesogloea in all sponges. Chitin apparently does not occur as a supporting structure in this phylum.

Among echinoderms collagen forms the bulk of the abundant mesodermal connective tissue (Marks *et al.* 1949; Randall, Fraser, Jackson, Martin & North, 1952). As in Porifera, chitin does not seem to occur in Echinodermata. These examples only contribute to our thesis in showing the abundant presence of collagen and the apparent absence of chitin.

NEMATODE WORMS

The cuticle of the readily available parasitic nematode, *Ascaris*, has been studied by several workers who define a principal constituent as a kind of collagen. The collagen fraction was first indicated by Chitwood (1936), who also found a number of different substances described as mucoid, fibroid and keratin (Chitwood & Chitwood, 1937). X-ray evidence confirmed the presence of collagen (Fauré-Fremiet & Garrault, 1944; Picken, Pryor & Swann, 1947), and this component must be localized at least principally in the obvious fibrils which cross one another at $\sim 135°$, for X-ray photographs with the beam perpendicular to the surface give two collagen diagrams crossing at this angle. But the total constitution of the cuticle is most complex.

Beneath the stout cuticle the epidermis, a syncytium, contains numerous fibrils looked on as supporting structures (Chitwood & Chitwood, 1937). X-ray photographs of the longitudinal chords give well-defined and detailed diagrams of the α-keratin type. These 'intracellular' supporting fibrils are not related to collagen but closely recall the characteristic intracellular tonofibrils of the vertebrate epidermis (Rudall, 1947). Thus, in *Ascaris* the epidermis is capable of synthesizing internally α-keratin or myosin-like fibrils and at the same time secreting collagen-type molecules. This situation is apparently met with again in the vertebrate epidermis, for which Porter (1954) describes the collagenous basement membrane as originating within the proximal parts of the epidermal cells, which of course also produce α-keratin-type tonofibrils.

Among nematodes chitin is absent except in the middle membrane of the egg shell, Hyman (1951). Dr C. A. Hopkins kindly provided me with dissected male spicules of *Ascaris* and with quantities of *Ascaris* eggs. On X-ray examination the male spicules did not show the chitin structure and

they rapidly disintegrated when heated in dilute alkali. On the other hand, the purified egg membranes did show the chitin structure but because of the low crystallinity and the poor orientation a detailed definition of the type of chitin was not possible. However, the chitin of the egg shell seems to be produced from within the egg itself, and not from any part of the genital tract, so in the nematode body we are not concerned with any effect of the presence of chitin on collagen production.

According to the Chitwoods's account the connective tissue of nematodes is unusual. These pseudocoelomic membranes and mesenteries warrant detailed study in bringing to light the full range of variation in collagen-containing structures. The basal lamella of the intestine of *Ascaris* is readily available, and this thin glassy external membrane was isolated by digesting away the intestinal epithelium in cold trypsin. It gives a typical wide-angle collagen diffraction pattern, and under the electron microscope is seen to be composed of three main layers (Pl. 1, fig. 1). Internally and externally there are thin membranes of very fine fibre and particulate texture, while the middle layer forming the bulk of the structure is smoother in texture. In none of these layers was a banded collagen structure observed nor could characteristic fibrils be detected which were likely to be fibrils of collagen. Much of the basal lamella may be secreted by the intestinal epithelium.

In *Ascaris* the bulk of the collagen of the body must exist in the external cuticle; the crossed fibrils of this do not appear banded in electron microscopic studies (shadow casts of thin stripped layers of *Ascaris* cuticle are shown in Pl. 1, fig. 2). Internally the only collagen examined has been that of the basal lamella which may possibly be grouped with the collagen of the cuticle in that mesenchyme cells may not be involved in its production.

MOLLUSCA

Collagen is very abundant in molluscs and can be identified readily in the lateral wall of the foot of *Patella*, indistinctly in the odontophoral cartilages of *Arion* and very clearly in the nuchal cartilage of *Loligo*. Where the gill lamellae of *Mytilus* were digested in cold trypsin the whole framework of the gill filaments remains intact, and this is mostly collagen according to X-ray diffraction patterns (see also Brown, 1952).

Chitin is commonly present in molluscs but is localized rather than continuously spread as in arthropods. It is present in the radula and buccal cuticle, and these structures in *Arion* give moderately crystalline α-chitin after purification in hot 5% KOH solution. The chitin of the very long radula of *Patella* is similar. The cephalopods are of special interest because of the remarkable observation by Lotmar & Picken (1950) that the chitin

of the huge skeletal 'pen' differs from the well-known chitin of the arthropods. They defined the new diffraction pattern given by the pen of *Loligo* and they called this structure β-chitin, believing it to be another crystalline form of polyacetylglucosamine. The main support for this conclusion was that the α-chitin pattern (arthropod type) appeared when the β-chitin was treated with 45% fuming nitric acid.

Chitin is quite extensively present in *Loligo*—the other regions where quantities are found are the large beaks, the radula and as cuticles lining the oesophagus and stomach (see Bidder, 1950). All these structures on purification give the α-chitin diagram, though not as outstandingly crystalline as in the case of arthropod cuticle chitin, while the *c*-axis period is about 2% larger. The purified chitins of the beak and of the pen dissolve readily in strong formic acid, and when the formic acid is evaporated a new diagram of a formic acid compound of chitin is obtained. On removal of bound formic acid by treatment in dilute mineral acid and alkali the α-chitin pattern is given by both specimens. Chitin from purified blow-fly puparia does not dissolve in formic acid under the same conditions, and the difference in the behaviour may be due to the more crystalline nature of the chitin in arthropod cuticles.

Thus in cephalopods two types of chitin are produced, namely, α-chitin in the beaks, radula and gut linings, and β-chitin in the pen. (The decalcified skeleton of *Sepia* is also a β-chitin.) In passing it may be noted that the horny material constituting the sucker rings of the tentacles does not appear to be a chitin. This material becomes a sticky mass when boiled in water, it disintegrates rapidly in hot dilute alkali and does not give the chitin X-ray diagram.

ANNELIDA

The epidermal system produces a more or less thick cuticle, thick in *Aphrodite* and thin in earthworms, and this cuticle is predominantly collagen in structure. The cuticle lines the external body completely and internally covers the fore-gut and hind-gut as a much thinner membrane. Though no defined dermis is present in earthworms a considerable collagen framework invests the system of muscles and can be isolated by digesting the muscle tissue away in cold trypsin.

Chitinous structures are present in the chaetae, and Lotmar & Picken (1950) first defined these as the new β-chitin structure in the case of the notopodal chaetae of *Aphrodite*. For the present purpose the chaetae of earthworms were isolated, purified in hot dilute alkali and partially oriented in a parallel array. The diffraction patterns were closely similar to those of β-chitin, and while they cannot be claimed as identical, they are much more

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akin to them than to α-chitin. In earthworms there is a thick chitinous cuticle lining the gizzard, and this stiff cuticle has been oriented by stretching, and X-ray photographs taken. Before purification the diffraction pattern is remarkably like that of the earthworm chaetae, but less like it as a result of purification in hot dilute alkali. But a pattern similar to α-chitin was not obtained, and the product is far from crystalline. There is close similarity between the earthworm chaetae and those of *Aphrodite*, but identity is certainly not established. The β-chitin in the polychaete is highly stable to heating in dilute alkali, while the similar structure in the oligochaete is much less stable in the same treatment. Of significance is the fact that the annelid chitins all differ from the typical arthropod chitin.

ONYCHOPHORA

Peripatus is noteworthy for possessing a robust dermis external to the muscle layers and just beneath the epidermis. This dermis is mainly a thick layer of collagen, as was readily demonstrated by X-ray photographs. External to the epidermis is a very thin cuticle, which Lotmar & Picken (1950) describe as giving a particularly sharp diffraction pattern which they judged to be α-chitin. I have examined the isolated but non-purified cuticle of *Peripatoides*, and though the cuticle proteins were not removed the structure bore distinct resemblances to α-chitin in a similar state of purity. The jaws also resemble the α-structure, while the tiny claws have not been examined. The tracheae of *Peripatus* are probably too fine in diameter to possess a chitinous intima. It is noteworthy that the cuticle of *Peripatus* is very thin compared with that of arthropods of similar size, such as caterpillars, and this is associated most probably with the development of a relatively massive dermis in *Peripatus*.

ARTHROPODA

These are, of course, characterized by a thick cuticle composed of chitin and of protein which is non-collagenous in nature. The chitin in particular has been widely studied and can be readily isolated in a relatively pure form. Everything points to the structure being polyacetylglucosamine though rarely is the nitrogen content found to be as high as the theoretical value of 6·9%. There is, therefore, uncertainty as to the detailed composition, as the commonly obtained value of about 6·4–6·5% may indicate the presence of non-nitrogenous impurities, substitution of —OH groups or the presence of a few non-amino sugars. On the other hand, values of 6·9% may be obtained if some de-acetylation has occurred. Nevertheless, the X-ray

diagram which was defined by Meyer & Pankow (1935) for lobster tendon is found in all other arthropod chitins examined, independently of the exact value of the nitrogen content. No diffraction data are available for gut linings, tracheae, peritrophic membranes or for such specialized regions as lens cuticle.

Estimates of collagen in arthropods are perhaps of special interest. Schneider (1902) describes the connective tissues of *Astacus* in terms of three orders of Leydig cells, and suggests that the structural fibrils of the first two orders are intracellular or at least closely applied to the cell surface, while the third-order cells produce masses of extracellular fibres much as in the case of typical fibroblasts. In cross-sections of the posterior intestine of *Astacus*, the least fibrogenic cells, first-order Leydig cells, are immediately beneath the cuticle-bearing epithelium while farther away from the epithelium are the second-order cells, with the frankly fibrogenic third-order cells lining the blood vessels. I have observed a similar situation in the tissue underlying the epithelium of the peduncle in *Lepas*. These examples suggest that collagen production is restricted nearest the cuticle-bearing epithelium while farther away from it collagen production becomes more evident. An apparent exception is to be found in the 'tendon'-muscle junction of the claw muscles of *Astacus*. This localized group of structures must be examined carefully to determine the nature of the chitin at that point as well as the specialization of the epithelium to muscle junction.

X-ray studies have shown that in the subcuticular tissue of the lobster and of the peduncle of the barnacle, *Lepas*, the wide-angle pattern of collagen is weakly present, while in their study of crab nerve Schmitt, Bear & Clark (1935) were able to define the presence of collagen. Collagen is thus not in doubt as a connective-tissue element in crustaceans. In the case of insects, Richards (1951) gives some account of the scarcity of connective tissue in discussing the basement membrane of the body wall. I have found much difficulty in demonstrating the presence of collagen in insect tissues. Nevertheless, in studies of large mantids I have obtained indications of the presence of collagen in the ventral nerve cord, while after isolating the stable tissue elements of these insects by treatment in saturated boric acid, followed by digestion in cold trypsin for several days, definite quantities of collagen material were established. Quantitative estimates are not possible at this stage though the evidence is in favour of relatively small quantities of collagen in the insect body. Richards (1951) describes concentrations of fibrous tissue beneath the cuticle of the proventriculus wall of *Corydalus cornutus*. In a preliminary examination of this material, the chitin was defined as of the α-type, while the presence of a collagen diffraction pattern, arising from the fibrous material beneath the

epithelium of the large ridges, has not been detected. Matheson (1912), in his original study, found that this tissue did not stain in the same manner as vertebrate connective-tissue fibres. As such tissue is probably formed by the cuticle-bearing epithelium it constitutes a crucial case for testing the point whether collagen and α-chitin can be produced together from the same epithelial system.

BRACHIOPODA

In *Lingula* the bristles protruding from the mantle, and the cuticle of the peduncle or stalk, are very well-defined examples of stable β-chitin. In the bristles the chitin chains are highly oriented parallel to the bristle length, while the cuticle of the stalk can be stretched by at least 50% extension and well-oriented diffraction patterns obtained. In the stretched stalk cuticle from which epithelium and sub-epithelial tissues were removed there is, in addition to the β-chitin pattern, a well-defined meridional arc of spacing 2·86 Å. which could be the principal wide-angle fibre period of collagen. As fresh material becomes available the possible presence of collagen within the cuticle will be examined. It is important to know whether collagen and β-chitin can be intimately associated together as are the β-protein, arthropodin, and α-chitin in insect cuticles (Fraenkel & Rudall, 1947).

The structure of the wall of the peduncle in *Lingula* has been examined in parallel with a similar study of the peduncle wall of *Lepas*. In contrast to the situation in *Lepas*, the epithelium of the *Lingula* stalk is immediately above a dense layer of collagen, about 5 μ thick. Where the cuticle is thicker this basal layer of collagen is thicker. No cells have been detected in the collagen layer and it is possible that the epithelium secretes both the β-chitin of the cuticle and the collagen of the 'basement' membrane. Considerable further investigation of this system in *Lingula* is required. It resembles the situation in annelids where the epidermal system produces β-chitin and collagen in parallel. But in *Lingula* it seems possible that collagen and β-chitin are produced together from the same cells.

HEMICHORDATES AND PROCHORDATES

In these groups we have several peculiar structures such as the basement membrane and skeletal elements of enteropneusts, the coenocium of pterobranchs and the test of tunicates.

The acellular skeletal elements of *Glossobalanus carmensis* and of *Saccoglossus cambrensis* have been examined in material provided by Dr C. Burdon-Jones. The skeleton of the proboscis and the branchial skeleton

both show a typical collagen diffraction pattern; the cellular chondroid tissue in association with the proboscis skeleton has not been examined. From fresh *Saccoglossus* specimens macerated in boric acid the collagen structure was readily defined in the 'basement' membrane. The main acellular skeletal elements, together with the 'basement' or limiting membrane, are regarded as being formed, in some places from epithelia, in others from mesenchyme cells, or again by the adhering secretion of mesenchyme and epithelial cells. These major collagenous elements thus occur as non-cellular cuticle-like structures. In marked contrast is the mesodermal chondroid tissue which resembles the arrangement in vertebrate cartilage—with numerous cells fixed in a fibrous matrix.

Also by the kindness of Dr Burdon-Jones there was an opportunity to examine the coenocium of *Rhabdopleura normani*. This is one of the structures commonly described as chitinous, but from preliminary studies it gives no evidence of being so. It disintegrates rapidly in boiling dilute alkali, while in X-ray examination a chitin pattern was not found.

The tunicates are of special interest in possessing a massive exoskeleton or test composed largely of 'native' cellulose and protein, the test being invaded by vessels continuous with the blood vascular system. Internally, the pharynx does not possess a branchial skeleton such as is found in enteropneusts on the one hand and in cephalochords on the other. The bodies of *Ciona intestinalis* were removed from the tests and were digested in cold trypsin solution for several days. In this way most of the muscle was removed while the body wall and mesenchyme membranes remained intact. From diffraction patterns of these residues we were able to conclude that collagen-type molecules were plentifully present. Thus we could dispose of the possibility that the great production of cellulose in the formation of the test had the effect of eliminating the production of collagen. A point of interest for further study is that the collagen framework did not appear of a fibrous nature but rather as a series of continuous membranes, suggesting a structure based on minute collagenous elements embedded in a matrix not readily broken up by trypsin. The presence or absence of extracellular collagen immediately beneath the cellulose-producing epidermis is an outstanding point which remains to be examined.

DISCUSSION ON THE DISTRIBUTION OF COLLAGEN AND CHITIN

The vertebrates have not been considered in this review. They form a comparatively uniform group of animals, usually of large size and in which collagen is always most abundantly present. But among the dermal

collagens, alone, there are marked differences in properties as evidenced by the lower thermal stabilities of various fish collagens compared with the much higher thermal stability of mammalian skin collagen (Gustavson, 1953). Whereas Gustavson gives evidence to suggest the importance of the hydroxyproline content in determining the molecular stability, Jackson (1953) describes the stabilizing effect of chondroitin sulphate so that the variable presence of such polysaccharides should be considered, as well, in evaluating the temperature stability of a given collagenous tissue. As distinct from invertebrates no chitinous structure is known to occur in any vertebrate tissue. However, throughout the vertebrates there is abundant dermal collagen underlying an epithelium which produces tonofibrils of the α-keratin type. Perhaps an important feature in this association is the secretion from the epidermis of a collagenous basement membrane which may contribute a favourable environment for the production of dermal collagen. The situation with an epidermis producing α-keratin-type fibrils internally, and at the same time secreting collagen, is best illustrated by the epidermis-cuticle relationship in *Ascaris*. Here the collagen is produced externally and no apparent dermal collagen is formed between epidermis and muscle.

Collagen has been demonstrated by X-ray means in most invertebrate types, and queries as to its presence in hemichordate and prochordate types have been answered; where there was any reasonable doubt as to the presence of collagen it was tested for by X-ray means. Among the various animals there are two main situations where collagen may be reduced to a minimum. The first group is to be found among the hydrozoan coelenterates, while the second group is typified by the insects. In both these groups chitin generally forms an outstandingly high proportion of the total bulk of the animal.

Collagen is considered to be low or absent in hydrozoan polyps, in millipores and at least in the pneumatophore member of Siphonophora colonies. In a sense the Hydrozoa may be looked on as having either a chitin-producing generation—the polyp, or a collagen-producing generation—the medusa. This suggests that the epithelial cells cannot produce both collagen and chitin at the same time. Because of the poor orientation and the low crystallinity, the hydrozoan chitins have not as yet been satisfactorily defined by X-ray means. There may be important specific differences between the hydroid, millipore and siphonophore chitins. Leaving aside for the moment the exact nature of coelenterate chitin, the general rule is that collagen (mesogloea) and chitin are not produced together in this phylum. By contrast, the annelids, particularly polychaetes, show abundant collagen and chitin production from the same

epidermal system, collagen as a continuous cuticle and chitin in the form of chaetae from specialized epidermal cells. But the striking feature about the chitin here is that it possesses the unusual β-structure, while where collagenous cuticles are completely replaced by chitin, as in arthropods, the chitin has the α-form. This indicates that β-chitin and collagen are compatible together, while α-chitin and collagen may be incompatible. Another case of the presence of β-chitin occurs in the brachiopod, *Lingula*. Here the thick cuticle of the stalk shows the typical β-chitin structure and there is X-ray evidence that collagen may possibly occur within the cuticle along with the chitin. Furthermore, there is a substantial layer of collagen immediately beneath the epithelium and the nature of this collagen layer suggests that it is secreted by the cuticle-producing epithelium. Thus in both the annelid and brachiopod examples, collagen and β-chitin production are closely associated and may arise together from the one epidermal system. On this basis we could account for the lack of association of chitin and collagen in coelenterates by assuming that the chitin here is of the α-type or more nearly similar to α- than to β-chitin.

The other major occurrence of β-chitin is in the large skeletal pen of squids, but the situation is made complex in that the chitins of beak, radula, oesophagus and stomach are of the α-type. Here first of all we require to know the kind of protein which is intimately associated with the two kinds of chitin. But there may be some correlation with collagen production as follows. The body wall of *Loligo* is richly provided with dermal fibres and the β-chitin-producing epithelium immediately overlies this robust production of collagen. Postulating an influence of the epithelium on the adjacent tissues we can see that there is an association between active production of β-chitin in the 'pen' and of collagen in the substantial dermal tissue. The nature of the dermal tissue underlying the α-chitin regions in *Loligo* and other molluscs has yet to be examined in detail. Should these regions be deficient in collagen there would be support for the postulate that where β-chitin is produced the dermal collagen is abundant and where α-chitin is produced dermal collagen is restricted in amount.

The situation in arthropods, where the chitin is apparently uniformly of the α-type, supports, on the whole, the above postulate. The first subepithelial connective-tissue element is generally Leydig's first-order cells, followed by second-order cells, and neither of these elements actively produces extracellular fibrils; the amount of dermal collagen is not great. In the arthropods, where we have evidence that the protein associated with chitin in the cuticle is not collagen-like, we can conceive the possibility that the chitin-producing epithelium suppresses the development of extracellular collagen in the subcuticular tissue. In passing, it seems possible

that the first- and second-order Leydig cells may contain formed collagen fibrils intracellularly.

In the foregoing discussion we were concerned with situations where dermal collagen production is suppressed and epithelial production of α-chitin is most active. In contrast, the body wall of *Peripatus* shows the very active production of dermal collagen while there is comparatively little chitin production in the form of an unusually thin cuticle. This cuticle seems to contain the normal α-chitin which, it is thought, replaces collagen rather than is associated with it. In a general way, the position in *Peripatus* is consistent with the view that there is an inverse relation between α-chitin and collagen production. We should like to know more concerning the amount of collagen adjacent to the regions of more active chitin production, such as beneath the jaw and claw epithelia.

It might seem appropriate to explain any inverse relation between chitin and collagen production in terms of natural selection, and thus to look on the total skeletal needs of the animal as being satisfied by one structure or the other. The concept of a definable chemical mechanism rests on the data obtained so far showing that, in epidermal products, β-chitin and collagen occur together, while α-chitin is not associated with collagen. Experimental test of the chemical mechanism is possible. One difficulty of relying on the X-ray definition of α- and β-chitin is that stable β-chitin can be converted to α-chitin in the test-tube and it is just possible that this conversion may occur secondarily in natural tissues. We also know that some β-chitins are much less stable than others. Thus one of the prime objects in the further development of this study is the more accurate chemical definition of true α- and true β-chitin, together with an understanding of intermediate types. Compatibility of β-chitin and collagen production and incompatibility of α-chitin and collagen would not be the only factor determining the details of collagen distribution; but it may be one important factor operating over a very broad field.

THE BYSSUS APPARATUS OF LAMELLIBRANCHS

A special interest in the study of the byssus apparatus is that it gives a view of various products that are linked together. Other cases of a comparable nature are the several products which come together with collagen in the nematode cuticle, and such structures as the earthworm cocoon which does not give the collagen or chitin diffraction pattern although it is linked together with the production of these fibrous structures from the earthworm epidermis.

A collagen-type protein was discovered in the byssus threads of the edible

mussel by Champetier & Fauré-Fremiet (1938), and by high extension they obtained the characteristically oriented collagen pattern. In addition they considered that collagen was present in *Pinna nobilis* byssus, but Centola (1936) and Jackson, Kelly, North, Randall, Seeds, Watson & Williamson (1953) obtained different results, as did Mercer (1952) with *Subitopinna*. However, Brown (1952) found the characteristic collagen pattern in *Pinna* and *Mytilus* byssus threads, as had been originally described by the French workers. Using *Mytilus* byssus threads there has been more consistency of results as Randall *et al.* (1952), Jackson *et al.* (1953) and Brown (1952) for *M. edulis*, and Mercer (1952) for *M. planulatus*, found essentially the same diagram in the case of the formed threads. There is emphasis of one feature which is additional to the generally accepted wide-angle diffraction pattern of collagen. Jackson *et al.* describe this extra feature as an accentuation of the equatorial reflexion at 4·4 Å., while Mercer describes a sharp ring centred at 4·2 Å. Measurement of his published photograph indicates that this ring is much more nearly 4·5–4·6 Å. From my own photographs of the byssus threads of *M. edulis* I find undoubted variation in the diffraction pattern of different specimens. Sometimes the anomalous reflexion is essentially disoriented, while the collagen pattern is typically well oriented. And the anomalous reflexion is always, according to my measurement, 4·5–4·6 Å. Also in some unstretched and stretched samples of byssus threads I have found additional meridional reflexions of 10·6 and 5·3 Å., while in stretched samples at 70–100% extension a weak meridional reflexion at 3·3 Å. has been observed. In stretched samples there is usually increased equatorial orientation of the 4·6 Å. reflexion, while non-axial arcs of 3·7–3·8 Å. are undoubtedly present and would correspond to the 210 of β-keratin proteins (Astbury & Street, 1931). These additional reflexions suggest that there is another protein (or another phase of the collagen structure) in the byssus threads or indeed two additional proteins, one represented by the meridional arcs 5·3 and 10·6 Å., and the other by a β-type protein of axial repeat 3·3 Å. and typical backbone spacing of 4·5–4·6 Å. with 210 reflexions at 3·7–3·8 Å.

The great reversible elasticity of byssus threads (Brown, 1952) does not indicate a continuous collagen structure but rather suggests analogy with vertebrate elastic tissue. Here also there is a collagen component, and where this has been removed the concentrated elastin fraction gives X-ray diagrams of the same general kind as those associated with collagen in byssus threads (Astbury, 1940). The constitution of byssus threads seems to be most variable, and the evidence is in favour of two or more main proteins being present which would account for the distinctly non-collagen-like infra-red absorption, chemical constitution and elasticity.

Because collagen diffracts X-rays so strongly compared with many other natural proteins it may well be that the amount of collagen-type protein is not even 50% of the whole byssus thread.

THE BYSSUS STALK OR STEM

About ten years ago I observed that the byssus stem of *M. edulis*, as distinct from the attached threads, gives an elaborate X-ray fibre photograph based on an axial period of about 43 Å. It was surprising that Jackson *et al.* (1953) described the byssus stem as a poorly ordered structure, though I could agree with the absence of the 2·85 Å. reflexion. Perhaps surprising too was Mercer's observation that the byssus stem of *M. planulatus* could be identified as collagen. I have examined a large number of byssus stems of *M. edulis* obtained from different sources, and they all give the same characteristic diffraction pattern. The stem was obtained free from byssus threads by peeling these off by hand. As also noted by Brown (1952) the stem contracts rather violently in hot water to less than half its length and is readily extended again. The fresh stem may be extended in cold or hot water or other media and extensions of 90% have been obtained. At higher extensions in cold water the amount of reversible extension is about 30%. The orientation of the X-ray diagram improves only very little by stretching, but there is also some deterioration in the perfection of the diffraction pattern.

The pattern to be described is that obtained from unstretched or moderately stretched specimens of byssus axis. The meridional region shows numerous layer lines which are orders of about 43 Å., all orders up to 16 being clearly visible except the 15th (2·86 Å.), which has not been observed. Certain orders are notably stronger, such as 4, 8, 10, 11, 12 and 16. There is a broad diffuse region of more intense reflexion on the meridian extending between the tenth and twelfth layer lines. A distinct second order of this band is clearly visible at wider angles as a comparatively strong and broad arc of spacing about 2·05 Å. At wider angles still there is a maximum about 1·11 Å. which doubtless arises from the sequence of —C—N—C— atoms in the polypeptide chains (Bamford, Brown, Hanby & Trotter, 1953). The amino-acid repeat should then be about 3·33 Å., and the nearest reflexion to this is the thirteenth order of 43 Å. at about 3·3 Å. Most of the axial reflexions are meridional or near meridional with the probable exception of those on the first layer line. Compared with the meridional series other reflexions are not so distinct; the most important are the two equatorial reflexions, moderately strong and moderately sharp at 12–12·3 Å., and the most diffuse spots about 4·5 Å. A number of other reflexions are visible but are far from easy to measure (Pl. 2).

On drying the stem in cold or hot ethanol and evaporating the alcohol, the diffraction pattern has become practically amorphous, but the characteristic meridional and equatorial reflexions return on washing the specimen in water. On drying by heating the specimens in vacuum at 65° C. over P_2O_5, most of the meridional reflexions disappear, except the fourth order which now has a spacing of 10·1 Å. On keeping in moist air the dry pattern returns approximately to the normal pattern. A comparison of the main reflexions, in the 'dry' and in the re-moistened specimen photographed at room humidity, is given in Table 1.

Table 1

Humidity	Fibre axis *A*	Equatorial *B*	Equatorial *C*
Dry	10·1 Å.	11·5 Å.	4·7 Å.
Room humidity	10·6 Å.	12·9 Å.	4·5 Å.

Water seems to be very important in stabilizing the structure along the fibre axis. Diffraction patterns with the byssus stem wet in water show the meridional reflexions to be well developed, while the equatorial reflexion *B* of Table 1 is spread into the central spot as occurs with collagen flooded with water (Astbury, 1940).

The type of diffraction pattern from the byssus stem bears a similarity to that of collagen in the presence of the diffuse equatorial reflexion at about 4·5 Å. and the much better defined reflexion at about 12 Å. Similar too is the way in which this spacing shows wide variations according to the water content. In preliminary infra-red examination, byssus stem (unstretched) shows a pronounced perpendicular dichroism (about 1·5), while the main NH stretching frequency is about the value normally obtained for α- and β-proteins. At present the structural feature of greatest interest would appear to be the axial period of 43 Å. (depending on the water content) associated with a comparatively fully extended β-protein. In the dry condition this repeat would seem to be about 40·4 Å. The thirteenth order of this is 3·1 Å., which is very close to the feather keratin axial period (Astbury & Marwick, 1932) as well as to the axial period obtained by Cowan, North & Randall (1953) for stretched collagen. But in contrast to feather keratin, the byssus stem does not show the denatured β-protein diagram as a result of boiling in water, and in this feature it is similar to collagen.

There are three readily definable molecular structures in the byssus apparatus of *Mytilus*. In the threads there is collagen and another structure which is tentatively regarded as a kind of β-protein. The third comparatively well-defined structure is located in the byssus stem. Some of this also appears to be present in the threads, as on occasions one obtains from

them the meridional reflexions of 10·6 and 5·3 Å. which are characteristic of the stem. We have never obtained the collagen diffraction pattern from the stem, though Mercer has apparently observed it in the stem of *M. planulatus*.

Smyth (1954) has made a notable contribution to our understanding of the formation of the byssus apparatus in *Mytilus*. A main part of the threads is shown to arise from the 'phenol' gland which directs its fibrous secretion into the distal extremity of the posterior groove. Is not this the main source of the oriented collagen fraction? Other material, mainly phenoloxidase, is contributed by the 'enzyme' gland, while the 'white' gland in its failure to give defined protein reactions is not regarded as contributing significantly to the byssus threads. We have digested the whole foot in trypsin at 37° C. and have noted that the tissues disintegrate except for the white gland which remains intact for several days. This resistance to proteolytic digestion is at least consistent with the lack of protein reactions as seen in histochemical studies. The white gland at its proximal regions is regarded as giving rise to the byssus stem; this stem is largely protein as seen from its infra-red absorption, while the nitrogen content is 14·5% and sulphur content 1·5%. Like the white gland the opaque proximal parts of the byssus stem are not readily digested in trypsin. It would seem reasonable to assign the collagen fraction as originating in the 'phenol' gland of Smyth, while the white gland, proximally, gives rise to the characteristic structure of the stem, and more distally contributes a component to the threads.

While all authors have found collagen in the threads of *Mytilus* byssus, the presence of collagen in *Pinna* byssus is doubtful. Collagen has been claimed to be present in *Pinna* byssus by Champetier & Fauré-Fremiet (1938) and by Brown (1952), while Mercer (1952), Jackson *et al.* (1953) and our observations on *P. vexillum* and *P. nobilis* have not revealed the presence of collagen. Lucas, Shaw & Smith (1955) have considered that the X-ray diagram of *P. nobilis* byssus resembles in some respects that of collagen, but do not find the 2·86 Å. reflexion. If there is sodium chloride present, giving a reflexion at 2·81 Å., the composite diagram could well suggest the presence of collagen; possibly all statements that the typical collagen diffration pattern is obtained from *Pinna* byssus, or the stem of *Mytilus* byssus, can be accounted for in this way.

Our X-ray photographs of the byssus of *Pinna vexillum* and *P. nobilis* show that the structure is essentially the same as that recorded in Pl. 2 for the stem of *Mytilus* byssus. The bulky stem of *Mytilus* byssus is little darkened and remains comparatively soft and flexible except at the peripheral layers of the most distal part. If one takes diffraction patterns of

the soft fine-fibred proximal regions of *Pinna* byssus, one obtains photographs almost identical with those of the *Mytilus* stem, save for the different relative intensity of the various orders on the meridian. In the hardened distal regions of *Pinna* byssus the diffraction pattern is altered somewhat, presumably due to the effect of phenolic tanning. This is understandable, for, as described above, the type of structure characteristic of *Mytilus* stem is markedly affected even by water, and would be variously distorted by the presence of tanning molecules. It is clear from these studies that in *Pinna* the whole byssus corresponds to only the stem of *Mytilus* byssus, the equivalent of the *Mytilus* threads being absent.

THE STRUCTURE OF β-CHITIN

There are some grounds for believing that the β-chitin structure is more closely associated with collagen, while the α-chitin structure replaces collagen. Thus all matters concerning the structure of the two kinds of chitin and the proteins associated with them become of special interest. For the present we can only review the physical structure of β-chitin, while the nature of the sugars involved is not as yet thoroughly investigated.

In their note announcing the discovery of β-chitin Lotmar & Picken (1950) define the main axial periods as 9·32, 10·17 and 22·15 Å. and draw attention to the shorter fibre-axis period as compared with that in lobster tendon chitin. Details of the β-chitin pattern are considerably less well defined than in the case of α-chitin. In our studies of purified *Aphrodite* bristles it was noted that a much modified diffraction pattern was obtained from the thoroughly dry β-chitin. The method of drying to produce this change was evacuation at 65° C. over P_2O_5 for 24 hr., while essentially the same effect is produced *in vacuo* over P_2O_5 at room temperature. Diffraction patterns taken under improved conditions of resolution will be necessary before the cell of these structures can be determined. There are significant differences between the patterns obtained at room humidity for various β-chitin samples depending principally on the extent of alkali purification; subsequent treatments with N-HCl or N-H_2SO_4 have clarified the patterns. A summary of the principal axial spacings for the 'room humidity' and the 'dry' β-chitin are given in Table 2.

The new moderately strong reflexions at 23·5 and 26 Å. for the dry and the room humidity specimens are somewhat larger than the possible unit cell dimensions of 20·7 and 22·5 Å. respectively, but are nearly related by the factor $\frac{2}{\sqrt{3}} \times c$ axis = new spacing. The new spacing changes with the water content in a similar way to the supposed 002 of the β-chitin structure,

thus supporting the view that all the reflexions are part of the same crystalline system. The type of X-ray diagram obtained for dry β-chitin is shown in Pl. 3, while a diagram of the room humidity structure is essentially the same as that illustrated by Picken (1949, fig. 57), minus the extra reflexions he describes (see also Picken & Lotmar, 1950). More highly resolved photographs of well-dried *Aphrodite* chitin indicate a monoclinic cell with β about 67° and giving a cell volume some 5% greater than that of α-chitin.

Table 2

	Room humidity (Å.)	Dry (Å.)
Meridian	10·1, 5·1, 2·55 W S M	10·1, 5·1, 2·55 S S M
Equator	26, 11·1–11·3, 4·7, 4·3, 2·36 M S S M M	23·5, 10·35, 4·75, 4·5, 2·38 M S M S M
(032)	3·29 M	3·25 M

W, weak; M, moderate; S, strong.

A preliminary infra-red examination of the dry β-chitin by Dr K. D. Parker has shown similarity and differences as compared with an earlier study of α-chitin (Darmon & Rudall, 1950). The two structures are entirely similar in the types of frequencies observed and in their dichroisms. The same evidence was obtained for free NH and free or weakly bonded OH groups; the carbonyl frequencies were again in two groups. All these things indicate that the symmetry of the chains as well as the chemical groups are similar in the two structures. The differences were in the shift of the NH stretching frequency of β-chitin to higher values, while the second CO frequency at 1625 cm^{-1} has also shifted towards the band at 1656 cm^{-1}. The shift of the NH stretching frequency to higher values may indicate less tight bonding and may be related to the greater cell volume which is probable in β-chitin. The shift in frequency is of the same general kind as that observed in collagen as compared with most fibrous proteins.

The β-chitin structure is thus recognizable as different from α-chitin on account of its diffraction pattern, the variation of this with the water content, and also because of its infra-red absorption spectrum. It is much less crystalline and is readily soluble in strong formic acid. (But some apparent α-chitins, such as *Loligo* beak, are also readily soluble in formic acid.) That the two structures are essentially similar is indicated by the apparent conversion of β- to α-chitin by solution in formic acid and by treating unpurified *Aphrodite* chaetae in 45% fuming nitric acid.

One is accustomed to different cellulose structures such as 'native' and 'mercerized', and in that case different bondings between OH groups seem

probable. In the case of chitin the CO—NH type bonding between the amino-acetyl side-chains would seem to be the dominating feature in determining the association of chains, but the type of bonding described for α-chitin (Darmon & Rudall, 1950) seems to fit equally well for β-chitin. There is no evidence for additional substitution of —OH groups. In order to determine the structure of β-chitin a closer study is required of the infra-red spectrum, the diffraction patterns and the density, while on the chemical side accurate determinations of nitrogen are needed together with knowledge of the sugars present.

GENERAL SUMMARY

1. The two principal supporting tissues which assist in holding together the cellular structure of animals are the collagenous system and the chitinous system.

2. These are generally of quite different origin, the collagen of mesodermal origin being internal and the chitin of ectodermal origin being external. In cuticles the chitinous system and the collagenous system replace one another as is seen when comparing the cuticles of arthropods with those of nematodes and annelids.

3. Among coelenterates the chitinous system is dominant in hydrozoan polyps, millipores and in the pneumatophores of Siphonophora. In these cases collagen may be absent or present only in minute quantity. On the other hand, in hydrozoan medusae, Scyphozoa and Anthozoa, chitin is absent while collagen is strikingly abundant.

4. The chitin of coelenterates does not appear to be identical with that of arthropods. Among cephalopods α-chitin is present in the radula, beak and in the cuticles of the oesphagus and stomach, while β-chitin is restricted to the skeletal pen.

5. The β-chitin structure appears to be associated with collagen-type cuticles, while the α-chitin structure replaces collagen-type cuticles. A preliminary account is given of the physical and chemical properties of β-chitin.

6. Problems concerning the presence of collagen in the byssus of lamellibranchs are discussed and the type of diffraction pattern occurring in the byssus stem of *Mytilus edulis* is described.

7. Other features considered include the skeletal elements of enteropneusts, the coenocium of pterobranchs and the presence of collagen in tunicates.

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PLATE I

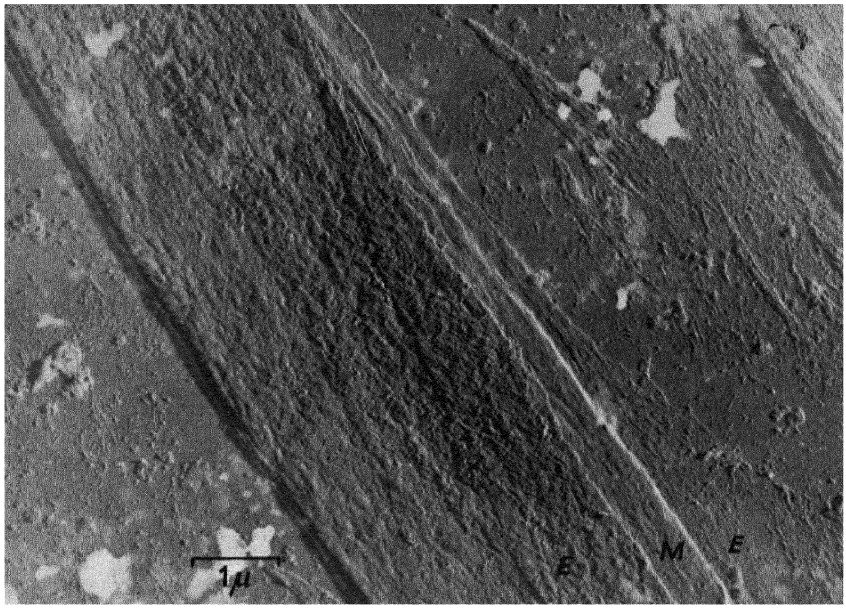

Fig. 1.

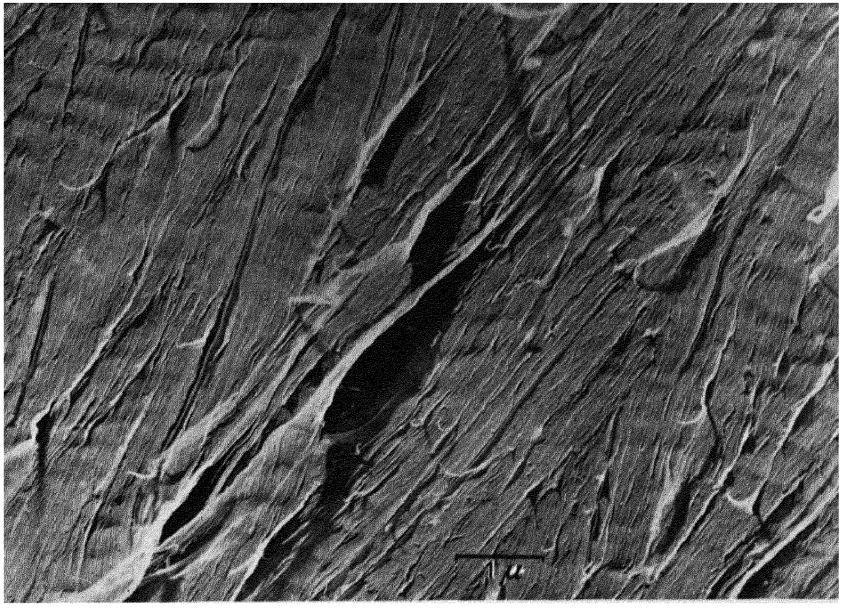

Fig. 2.

For explanation see p. 71

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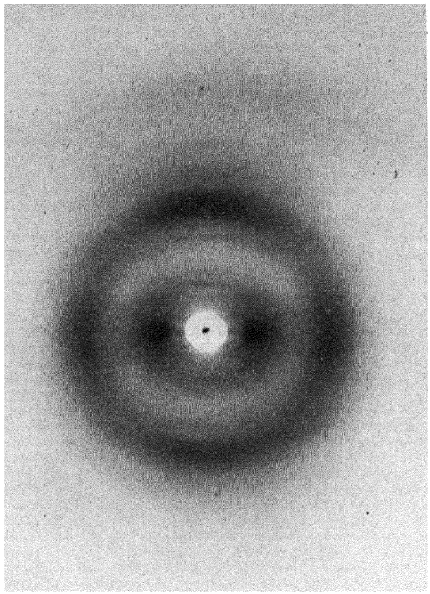

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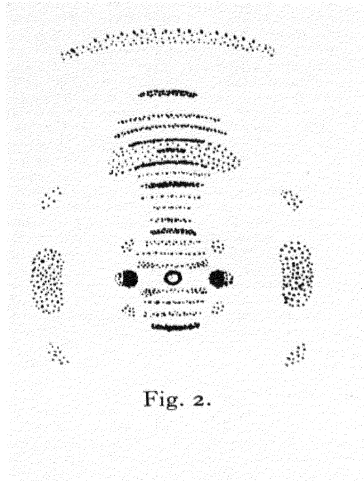

Fig. 2.

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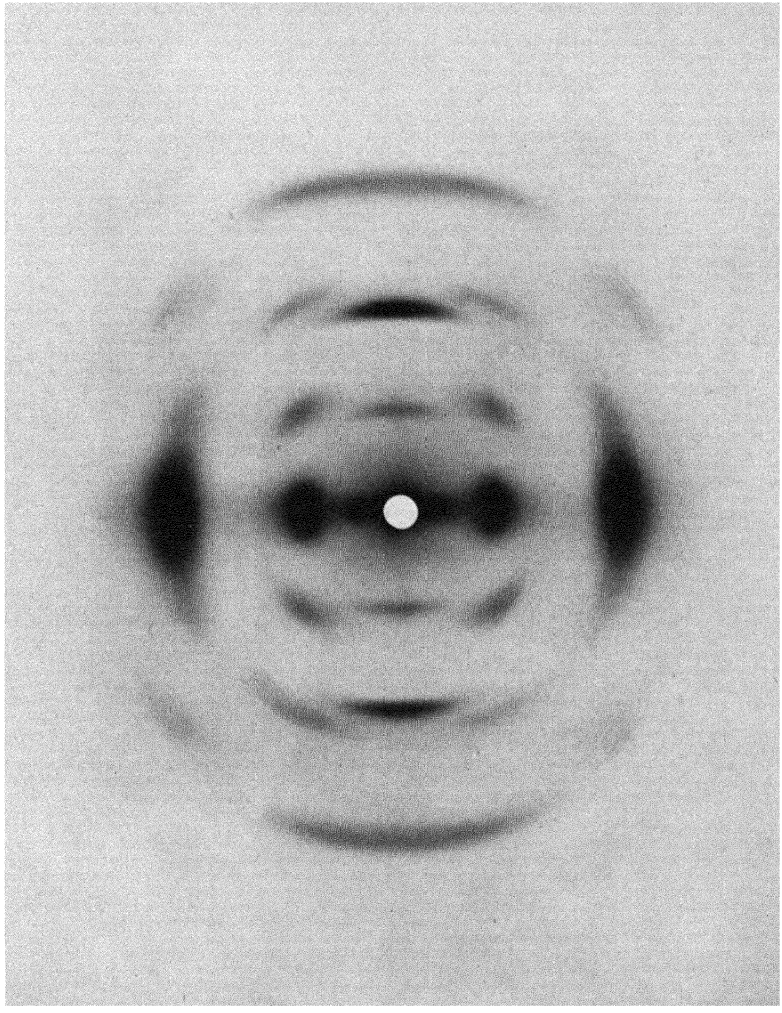
PLATE 3



For explanation see p. 71

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Electron micrograph. Basal lamella of intestine of *Ascaris*. Shadow-cast with gold-palladium; replica. *E* = external layers; *M* = middle layer.

Fig. 2. Electron micrograph. Cuticular fibrils of *Ascaris*. Shadow-cast with gold-palladium; replica.

PLATE 2

Fig. 1. X-ray photograph of byssus stem of *Mytilus edulis*. Taken in cylindrical camera (3 cm. radius) with fibre axis tilted 10°. Room humidity.

Fig. 2. Diagram illustrating the main features of Fig. 1.

PLATE 3

X-ray photograph of purified *Aphrodite* chaetae; specimen dried *in vacuo* over P_2O_5 at 65° C.

METABOLISM OF COLLAGEN UNDER NORMAL CONDITIONS

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DEFINITION AND GENERAL PROPERTIES OF COLLAGENS

The collagens are clearly distinguished from all other proteins by a number of well-defined biological, physical and chemical properties. Collagen is found in tissues mainly or entirely in the form of fibres which can be recognized by their general appearance and by their rather unspecific affinities for certain acidic dyes. The fibres show marked swelling on exposure to acids and alkalis and are resistant in their native state to trypsin, but not to pepsin; on prolonged heating with water they are converted to gelatin. Collagen is a major component of mesenchymal tissue and is therefore of mesodermal origin. Using these criteria collagen has been identified in a large number of tissues of all vertebrates examined and in many invertebrates except Protozoa. Collagen-like proteins have not been found in micro-organisms or higher plants.

More recently collagens have been characterized by their distinctive physical properties as revealed by X-ray diffraction and electron-optical methods. It was Astbury (1938) who first distinguished clearly the collagen group of fibrous proteins from the keratin-myosin-epidermis-fibrinogen (k.-m.-e.-f.) group and the extensive recent work, in which both high- and low-angle diffraction was used, has established valuable criteria for the recognition of collagens (for review see Bear, 1952). But the physical tool which has been most useful lately is the electron microscope, and the characteristic pictures it produces have been widely employed for the recognition of collagen fibres.

On hydrolysis the collagen yields a 'spectrum' of amino-acids which differs sharply from that found with any other class of proteins. In Table 1 analyses with respect to some amino-acids are given for two fairly typical and well-characterized proteins, of the albumin and globulin class respectively, and for mammalian tendon collagen and for elastin. It can be seen that collagen is quite exceptional in yielding very large amounts of glycine and proline, very small amounts of histidine and undetectable quantities of tryptophan and cystine. The small amounts of tyrosine which have been found in almost all collagen samples analysed may be due to contamination by non-collagenous proteins. Hydroxyproline and hydroxylysine have so

far not been identified in hydrolysates of other groups of protein with the exception of elastin for which values of 1·7–2·2% of hydroxyproline have been reported. However, Bowes & Kenten (1949), using a chromatographic method, could not detect hydroxyproline in a hydrolysate of purified elastin, and it is possible that the small quantities of this imino-acid found in other preparations are due to incomplete removal of collagen.

Table 1. *Comparison of the amino-acid composition of mammalian collagen, elastin, egg albumin and γ-globulin*

The values are given as g. amino-acid/g. dry protein and are mainly taken from the compilations of Tristram (1953) and of Bowes & Kenten (1949).

	Collagen	Elastin	Egg albumin	Human γ-globulin
Glycine	26·5–27·2	25·7–29·4	3·05	4·2
Proline	14·8–15·1	15·2–15·6	3·60	8·1
Tyrosine	0·3–1·1	0·25–1·4	3·68	6·8
Tryptophan	0	0	1·20	2·9
Cystine, cysteine	0	0·2–0·6	1·86	3·1
Arginine	8·5	0·3–1·1	5·72	4·8
Histidine	0·7	0	2·35	2·5
Lysine	4·5–4·6	0·0–0·5	6·30	8·1
Aspartic acid	6·3–6·7	0·0–0·6	9·30	8·8
Glutamic acid	11·3	2·7–3·3	16·50	11·8
Hydroxyproline	14·0–14·5	0·0–2·0	0	0
Hydroxylysine	1·1–1·2	0 (?)	0	0

In this connexion it is of interest to note that *allo*-L-hydroxyproline, a stereo-isomer of the L-hydroxyproline found in collagen, has been isolated from the hydrolysate of phalloidin, a toxic peptide produced by *Amanita phalloides* (Wieland & Witkop, 1940) and recently Giri, Gopalkrishnan, Radakrishnan & Vaidynathan (1953) have identified hydroxyproline, of so far undetermined steric configuration, in the extracts of leaves of the sandal-wood tree (*Santalum album*). It would thus appear that L-hydroxyproline (or its stereo-isomers) have a wider distribution in nature than collagen.

Both the similarities and dissimilarities in amino-acid composition between collagen and elastin are striking (Table 1). Both proteins contain almost identical and large amounts of glycine and of proline, are free from tryptophan and contain only traces of tyrosine. But, whilst elastin contains only very small quantities of basic amino-acids and of aspartic acid, and relatively little glutamic acid, the composition of collagen is quite 'normal' with respect to the 'polar' amino-acids arginine, lysine, aspartic acid and glutamic acid. It is tempting to speculate that these two proteins, which are so closely associated in connective tissue, are formed in the same cell and share peptides with the sequence -glycyl-prolyl- or -prolyl-glycyl- as intermediates. But such data on amino-acid sequences in collagen as are

available (Schroeder, 1953) do not support such a hypothesis, and the evidence is not conclusive.

There has been considerable disagreement as to whether the protein component of reticulin fibres is collagen. Reticulin occurs in lymph nodes, spleen and many other, especially embryonic, tissues and differs histologically from typical collagen fibres by being stainable with silver and by its greater resistance to heat treatment. But it appears to have the same amino-acid composition (Bowes & Kenten, 1949) and gives the typical collagen picture with the electron microscope (Irving & Tomlin, 1954). Available evidence suggests that the reticulin fibre, as observed by the histologist in a section, differs from the collagen fibre in containing relatively large amounts of polysaccharide in intimate association with the proteins. This carbohydrate appears to be responsible for the silver staining (see Kramer & Little, 1953) and possibly, in a manner as yet unexplained, for the great resistance to heat. The presence of polysaccharide in epithelial basement membranes which appear to be of the reticulin type, also explains the striking immunological reactions observed by Cruickshank & Hill (1953). However, removal of non-protein from the reticulin fibre produces material which is at present indistinguishable from collagen and is at least partly estimated as such by many of the methods used, particularly that of Neuman & Logan (1949).

DISTRIBUTION OF COLLAGEN IN MAMMALIAN TISSUES

Collagen is present in almost all tissues, but its concentration relative to those of other proteins varies within wide limits. To illustrate this point values have been collected from the literature and are shown in Table 2. Since the methods for the estimation of collagen are still not quite satisfactory, these figures are only approximate, but certain general conclusions clearly emerge: in tissues with a relatively low proportion of cells such as tendon, collagen forms about 80–95 % of the total protein. Rogers, Weidmann & Parkinson (1952) have shown that 90–95 % of the organic matrix of the bones of man, rabbit and ox consists of collagen or collagen-like proteins. In whole skin the proportion is lower and appears to vary between 55 and 75 %. The collagen contents reported for different muscles of various species lie between 1 and 3 % on a dry-weight basis (see, for example, Lampitt, Baker & Brown, 1952); the values seem to vary with the source of the muscle, but may also depend on the extent to which fasciae and tendons have been included in the sample analysed. The tissues of the intestinal tract and the lungs have collagen contents of well over 10 % (in terms of dry weight), whilst the value for liver is relatively low. It would

be wrong to assume that the collagen found in an organ is of either morphologically or metabolically uniform character. Thus liver collagen appears to be present in the peritoneal capsule, in the walls of the blood vessels and bile ducts and in their surroundings and also as a reticulin network in the walls of the sinusoids. Similarly, in skeletal muscle there is a large difference between the delicate reticular fibrils of the endomysium and the coarser fibres of the epimysium or perimysium. As Table 2 shows brain contains very little collagen, and the small amount found is probably mainly derived from the meninges and the blood vessels. It appears that the supporting function which is exercised in all other organs by connective tissue containing collagen and elastin fibres is discharged in the nervous system by the neuroglia.

Table 2. *Approximate collagen contents of some tissues of various species*

The values are taken from the papers by Neuman & Logan (1950) and by Lowry, Gilligan & Katersky (1941) and are expressed in terms of g. per 100 g. dry tissue (mostly fat-free). The papers of Neuman & Logan and Lowry *et al.* are referred to as (1) and (2) respectively.

Source	Collagen	Reference
Chordae tendineae: ox	84·6	(1)
pig	76·9	(1)
Tendo calcaneus: elephant	93	(2)
man	86	(2)
Skin: dog	64·3	(1)
guinea-pig	72·1	(1)
man	71·9	(1)
man	79	(2)
Bone: tibia of ox	24·2	(1)
femur of rat	15·1	(1)
Aorta: rat	31·0	(2)
ox	23·2	(1)
Liver: ox	2·0	(1)
pig	2·5	(1)
rat	0·64	(1)
man	0·86	(2)
Muscle: shoulder of ox	2·1	(1)
abdominal of rat	5·8	(1)
adductor of thigh: rat	1·0	(2)
Spleen: ox	3·1	(1)
pig	2·4	(1)
Lung, rat	11·3	(1)
Duodenum, rat	12·0	(1)
Stomach: cardia, rat	23·6	(1)
pylorus, rat	13·0	(1)
Brain: cerebrum, rat	0·22	(1)
rat	0·36	(2)

There is no quantitative information available about the total collagen content of any mammal, but it can be estimated that between 25 and 35% of the total protein of the body is collagen. This implies that about 70% of the total glycine and proline of the body and practically all the

hydroxyproline is contained in collagen. It is clear, therefore, that this fibrous protein is of great importance in the overall nitrogen metabolism and especially in the metabolism of the amino-acids glycine, proline and hydroxyproline.

CLASSICAL VIEWS ON EXTRACELLULAR MATTER

Even before isotope methods were used in studies of protein metabolism, it was generally assumed that collagen is somewhat inert. This belief was partly based on the resistance shown by collagen, in comparison with most other proteins, to acids, alkalis and some proteolytic enzymes. But a more important reason was probably the fact that collagen is found invariably outside the cell. When, more than 100 years ago, the cell theory became established through the efforts of Schleiden, Schwann, Virchow and others, it was accepted that cells were the only ultimate units of living matter and that life did not exist outside the cell. Virchow, for example, in his famous *Cellularpathologie*, subscribes to this opinion, although he admits that intercellular substance may be under the influence of living cells. This view has dominated morphological and physiological thinking for a long time, although it was challenged by some histologists such as Heidenhain, Hueck and Studnicka, who argued that this sharp distinction between intracellular and extracellular matter is artificial. Cameron has recently (1953) reviewed the subject and stressed the fact that arguments about the validity of the rather rigid cell theory as propounded, for example, by Virchow, have largely centred on the nature of extracellular matter. The extreme cellular view of Virchow was opposed by the 'symplastic' theory which stressed the structural continuity of living matter in Metazoa and denied the existence of any fundamental distinction between protoplasm and intercellular ground substance. Consideration of the arguments put forward by both sides leads the modern reader to the conclusion that the orthodox morphological approach to this problem is not very helpful, particularly if it is associated with a tendency to metaphysical speculation. As Cameron says, 'the application of modern chemical and physical methods of investigation is highly desirable if the problem is to be settled'.

Information on the composition of the intercellular phase is still rudimentary, largely owing to the difficulty of obtaining samples sufficiently large for chemical analysis and free from contamination by cellular material. However, the protein composition of tendon (Table 2) shows that the amount of non-collagenous protein in whole tendon is relatively small, and it would appear that most or all the protein which is not collagen is either material derived from cells or plasma protein which is always present in the

extracellular phase in significant amounts. There is the possibility that a specific protein is associated with the mucopolysaccharides present in tendon, but there is no evidence on this point. No comprehensive search for enzymes has been made in connective tissue, but it is almost certain that systems responsible for glycolysis, aerobic oxidation and phosphorylation are absent from the extracellular compartment, and it is probable therefore that no synthetic, energy-requiring processes take place to any extent outside cells. If self-reproduction is taken as the criterion of life, then extracellular matter is non-living. The work of Hastings and others (see, for example, Mannery, Danielson & Hastings, 1938) has shown that the extracellular phase of connective tissue resembles plasma in its content of inorganic ions. We may, on the basis of the facts at present available, consider the extracellular phase as a functional extension of plasma which acquires its specific character and gel-like consistency by the deposition of collagen and elastin fibres and the presence of hyaluronic acid and other polysaccharides.

It has been known for some time that the collagen, at least in some tissues, can be broken down under certain experimental conditions. Thus, Cameron & Karunaratne (1936), using histological methods, observed that the extensive fibrous bands formed in the livers of rats poisoned with carbon tetrachloride may again disappear if administration of toxic material is discontinued. These findings were confirmed by Morrione (1949), who measured quantitatively the changes in collagen content of the livers by a chemical method. Very striking changes in the collagen content of the uterus of the rat during pregnancy and lactation have recently been described by Harkness & Harkness (1954). These workers found a large increase in uterine collagen during pregnancy and a rapid fall during the involution of the organ. It is clear, therefore, that certain tissues can either reabsorb or break down collagen under the influence of physiological and pathological stimuli. However, methods which consist of measuring changes in collagen content cannot give quantitative information about the rate of 'turnover' of this protein under normal conditions. The use of isotopically labelled amino-acids appeared to provide a more promising approach to this problem.

THE USE OF ISOTOPE METHODS

It was Schoenheimer who first showed that isotopically labelled amino-acids are rapidly incorporated into various body proteins. This led to the concept that most or all proteins in higher animals are metabolically unstable and that constant renewal is a characteristic property of all living matter. However, a quantitative comparison of the rate of incorporation into the mixed proteins of various organs revealed the existence of large differences between

tissues (Schoenheimer, Ratner & Rittenberg, 1939); thus administration of leucine labelled with ^{15}N and deuterium produced relatively high isotope values in liver and plasma proteins and low values in skin and muscle. In these and the later experiments of Shemin & Rittenberg (1944), which also showed similarly large differences between liver and 'carcass', the proteins of whole organs were used, and thus no conclusions could be drawn about the behaviour of collagen, beyond the fact that its rate of turnover must be appreciably lower than of the proteins of plasma.

Table 3. *Comparison of specific radioactivities of the mixed proteins of livers and muscle with those of the collagen of tendon in old rats*

The figures represent average values obtained in many published and unpublished experiments with rats older than 6 months and body weights greater than 300 g. The animals had a dose of 10 μc. [$\alpha-^{14}C$] glycine/100 g. body weight. The radioactivities are expressed as counts/min./2 sq.cm. at infinite thickness of 2:4-dinitrophenylglycine.

Protein fraction	Average radioactivities at					
	1 day	1 week	2 weeks	3 weeks	5 weeks	7 weeks
Mixed liver proteins	2800	1250	620	360	150	120
Mixed muscle proteins	610	510	405	420	350	300
Tail tendon collagen	12	10	24	18	15	15

Our own early experiments (Neuberger, Perrone & Slack, 1951) were done with collagen obtained from the tail tendon of the rat. Glycine labelled with ^{14}C was given in one single dose to rats of various ages; the animals were killed after suitable intervals of time and tendon collagen and proteins from various organs were prepared. After hydrolysis glycine was isolated and its radioactivity was determined. It was observed that the radioactivity of the collagen glycine was mainly dependent on the age of the animals. With old rats, i.e. animals aged 6 months or more and with body weights of about 300 g., only minute amounts of radioactive glycine were incorporated into the tendon collagen. Table 3 shows that the maximum radioactivity of the glycine of the mixed liver proteins was about 100 times greater, and that of the glycine of the mixed proteins of muscle about 25 times greater than the maximum radioactivity of the tendon collagen glycine. It has been calculated that the mixed proteins of the rat liver have an average half-life of about 4–6 days, whilst for the proteins of rat muscle the corresponding value is between 20 and 35 days. No estimate of real quantitative significance of the half-life of tendon collagen can be given, but it would appear that, if the radioactivities observed represent an actual turnover, the half-life of the collagen of the tail tendon must be about 500 days or more.

With actively growing rats, having initial body weights ranging from 50 to 80 g., the results were completely different (Fig. 1). The radioactivity of the glycine of the mixed liver proteins fell sharply after the first day, and the value found at 50 days was only about 1 % of the maximum figure. The mixed proteins of muscle show an initial radioactivity slightly lower than those of liver, but the rate of decrease is smaller and had fallen at 50 days to about 6–7 % of the maximum figure. With tendon collagen the initial value was about 40 % lower than the corresponding figure for liver proteins, but even at 50 days the specific radioactivity had only decreased to 20–25 % of the maximum value.

The quantitative interpretation of these findings is difficult for the following reasons: the free glycine of the body which is the precursor of the protein glycine has a high isotope content immediately after the administration of the amino-acid; this falls, first sharply, then more slowly (Henriques, Henriques & Neuberger, to be published), and after about 10 days the isotope content of the free glycine is very small compared with the initial value, but still appreciable in comparison with that of the glycine in the body proteins at that time. This fact already complicates the interpretation of time-activity curves of proteins in terms of turnover values in the adult, but in the rapidly growing animal the position is even more difficult. If the labelled glycine is given, for example, to a weanling rat which may double its body weight in about 10 days, radioactive amino-acid will get incorporated both into labile proteins which lose their label again and into metabolically stable proteins in which the labelled amino-acid remains fixed. The glycine supplied in the diet or synthesized by the animal in the period following the feeding of the labelled compound will not be radioactive, and thus radioactive protein will get progressively diluted with new material representing net synthesis and having little or no radioactivity. This factor which reduces specific, but not total, radioactivity operates with both metabolically stable and labile proteins. On the other hand, the continuous renewal of cell substances occurs only with the labile protein fraction and reduces both specific and total radioactivity. The operation of both causes explains the large decrease in specific radioactivity found especially with liver, but also with muscle (Fig. 1). In the case of tendon collagen the decrease is mainly due to the 'growth dilution' phenomenon, and it is even possible that the small radioactivity found with old rats could be explained by a net small increase of proteins without any turnover. But it is more likely, for reasons to be discussed below, that there is a small rate of renewal of collagen in both young and old rats.

In later experiments (Neuberger & Slack, 1953) collagen was isolated from liver, bone and skin as well as from tendon, and the radioactivities of the

glycine samples were compared. The values for skin collagen were similar to those of tendon collagen, but the values for the collagen of the liver and particularly those of bone were appreciably higher. Thus, for old rats the average values of radioactivities (relative to tendon) of the glycine-collagen samples in a large number of experiments were as follows: tendon, 1·0; skin, 1·4; liver, 2·6; bone, 4·6. In another experiment rats aged 103 and 151 days and weighing between 260 and 320 g. were given radioactive glycine and killed 1 week later. The actually measured radioactivities of the collagen-glycine samples were as follows (average values): tendon, 32;

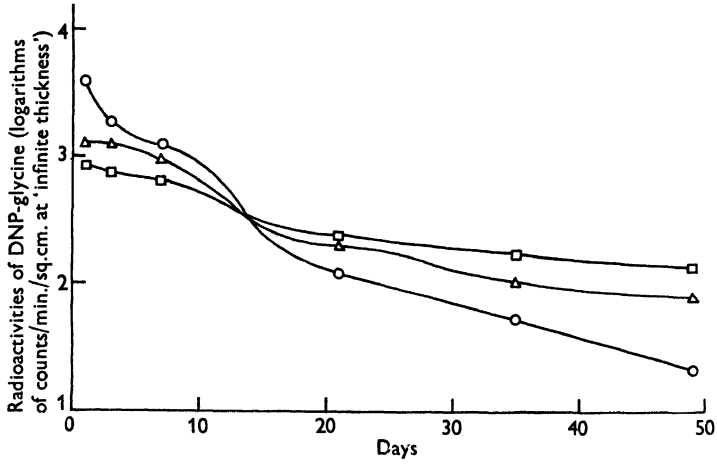

Fig. 1. Time-activity curves of the mixed proteins of liver (○), of the mixed proteins of the muscle (△) and of the tendon collagen (□), of young rapidly growing rats. The logarithms of the activities are average values from a number of published and unpublished experiments. The initial body weights of the rats were between 35 and 50 g. The radioactive glycine was given at day 0.

skin, 83; bone, 169; liver, 180. These values, though still small if compared with non-collagenous liver proteins or even muscle proteins which were about 1500 and 600 respectively, suggest that collagen is constantly being replaced, but that the rate of renewal varies with the tissue. We have suggested the possibility that in the young, growing animal the rate of renewal of collagen may be somewhat higher than in the adult, but the data available are not sufficient to decide this question. Results which in general are similar to ours have been reported by Robertson (1952) who administered ^{15}N-labelled glycine to guinea-pigs. It can be concluded that collagen, quantitatively the most important protein of the mammal, has a very slow rate of turnover and makes only a small contribution to the 'dynamic pool' of body proteins.

THE FORMATION OF COLLAGEN FIBRES

Numerous and often contradictory explanations of the mode of fibre formation in connective tissue have been given by histologists, but recently a fair amount of agreement on the main questions involved has been obtained. Thus, it is now almost universally believed that this process occurs extracellularly (see, for example, Doljanski & Roulet, 1933; Stearns, 1940), but the hypothesis, which was at one time favoured by histologists, that fibrin may be converted to collagen, has to be rejected on chemical grounds. Stearns (1940), who observed fibre formation in the rabbit's ear by means of a transparent chamber, concluded that fibroblasts secrete cytoplasmic material in a soluble form into the intercellular space, and that this material is then transformed into fibres by enzymes also supplied by the fibroblast. But it is also possible that the production of fibres is a physico-chemical process, akin to polymerization, or that it is initiated by factors produced by other cells, such as histiocytes, or transported to the connective tissue by the plasma.

If the production of fibres is indeed a conversion of a precursor to collagen occurring outside the cell, it would be expected that connective tissue in a state of proliferation should contain a protein resembling in its amino-acid composition ordinary collagen, but differing from the latter in solubility and molecular size or shape. It should also be possible to test with the aid of isotopes whether such a protein is indeed a precursor of collagen fibres.

It has been known for over 50 years (Zachariades, 1900) that a fraction of the collagen of the tail tendon of the rat was soluble in dilute acetic acid. The properties of this acid-soluble collagen have been investigated by Nageotte, Guyon, Fauré-Fremiet, Leplat and more recently by Russian workers, particularly Orekhovich. It was found that this protein, which was named procollagen by Orekhovich, can be reprecipitated in the form of fibres which are indistinguishable from those of native collagen, if examined with the electron microscope. This type of collagen is not soluble at a pH greater than 5 or 6. The existence of possible other species of collagen-like proteins, soluble at a neutral or alkaline pH, was suggested by the work of Highberger, Gross & Schmitt (1951). It appeared likely that one or both of these proteins might be the postulated precursor of fibrous collagen.

Our own investigations (Harkness, Marko, Muir & Neuberger, 1954) on this problem began with a general survey of skin proteins of young rabbits, using the amounts of tyrosine, hydroxyproline and glycine in the various protein fractions as guide. Most of the hydroxyproline was associated with

the insoluble fraction ('collagen proper'), but there was another large fraction which could be extracted with dilute acid, but not with alkali. The latter is the soluble collagen of Nageotte or the procollagen of Orekhovich. In addition, there was a third fraction, amounting to about 4% of the total collagen, which could be extracted with a neutral or alkaline buffer. This material, which we have called alkali-soluble collagen, lost its solubility at neutral or slightly alkaline pH during the process of purification which involved precipitation and dialysis at an acidic pH. The material finally

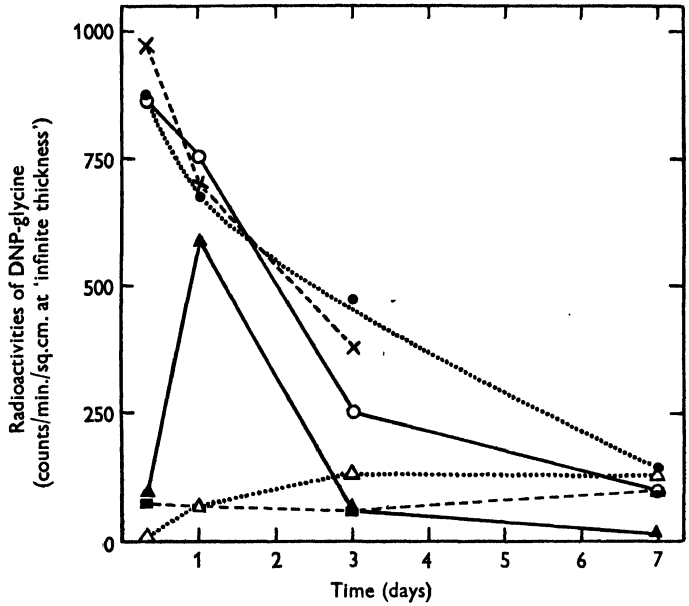

Fig. 2. Time-activity curves of proteins from the skin and plasma of rabbit. The averaged values of the radioactivities of a number of experiments have been used to plot the above curves: ▲, alkali-soluble collagen; △, acid-soluble collagen; ×, water-soluble protein (extracellular plasma protein of skin); ●, plasma proteins of plasma; ○, citrate-insoluble protein; ■, insoluble collagen. This figure is reproduced from the *Biochemical Journal* (56, 558, 1954) by permission of the Editors.

obtained resembled acid-soluble collagen in all the properties which we examined. It only differed from the latter in being extractable in its native state by neutral or slightly alkaline buffers.

However, metabolic experiments in which isotopically labelled glycine was used revealed profound differences between the two soluble collagens (Fig. 2). The rapid incorporation of isotope into the alkali-soluble fraction and the following rapid fall of radioactivity suggest strongly that this material is a true precursor of fibrous collagen, and the hypothesis may be put forward that alkali-soluble collagen is excreted by fibroblasts into the extracellular space and is there converted into fibres. It may be that this collagen-

like protein is originally associated with another substance, such as another protein or a polysaccharide, and that this combination is responsible for the greater initial solubility. But the two soluble collagens may differ only in particle weight. This question can only be decided when the alkali-soluble collagen is isolated in its native state. The possibility that the acid-soluble collagen is an intermediate between alkali-soluble and insoluble collagen cannot be completely excluded, but the data shown in Fig. 2 are not in favour of such a hypothesis. It is more likely that acid-soluble collagen is mainly derived from fibres recently laid down or from outer layers of older fibres.

The data shown in Fig. 2 also demonstrate the great difference in metabolic behaviour of the protein fractions obtained from the same organ. It would appear that the plasma proteins which are present in the extracellular compartment in significant amounts are metabolically as active as the plasma proteins found in blood. In addition, evidence was obtained that another protein fraction which we believe—without much evidence—to be derived from the cellular portion of the skin has a turnover of the same order as that found for liver, kidney and other glandular organs. It would thus appear that, whilst the insoluble collagen fibres are relatively inert, other, more soluble, proteins of connective tissue are being continuously broken down and resynthesized at fairly rapid rates.

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THE INFLUENCE OF COLLAGEN ON THE ORGANIZATION OF APATITE CRYSTALLITES IN BONE

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The X-ray diffraction data from compact bone is to be associated with two main structural components, collagen and the mineral salt, apatite. The wide-angle reflexions of these two components are readily distinguished, and in bone in which the collagen fibres are well aligned, the apatite crystallites are found to be oriented with their crystallographic c-axis parallel to the fibre axis of the collagen. The apatite unit cell is hexagonal, with $a = 9{\cdot}41$ Å. and $c = 6{\cdot}87$ Å., and the $00l$ reflexions are oriented at right angles to the 12–15 Å. side-spacing of the collagen.

In the intact bone, the apatite reflexions are poorly defined, mainly because of the small crystallite size. The widths of the wide-angle reflexions have, in fact, been used to obtain an estimate of the order of size of the crystallites (Stühler, 1938; Brandenberger & Schinz, 1945), and the most probable values seem to lie in the range 100–300 Å. In well-oriented wide-angle diffraction patterns, it is clear that the $00l$ reflexions are much sharper than reflexions from other planes, thus indicating an asymmetric crystallite with elongation along the c-axis. A crystallite dimension of 200 Å. has been estimated from the widths of the $00l$ reflexions alone (Jensen & Möller, 1948).

The apatite crystallite also gives an intense low-angle particle scatter which provides reliable information concerning its shape and size. Studies of the low-angle scatter from thin longitudinal- and cross-sections of compact human bone (femur) have shown the apatite particles to be approximately rod-shaped, with dimensions of 210×75 Å. (Engström & Finean, 1953; Finean & Engström, 1953). The form of the scattering curve indicates a very uniform particle size, and a very precise orientation of the long axis parallel to the collagen fibre axis. Long bones from other sources such as cow, hen and frog appear to have apatite crystallites of similar dimensions, the lengths in particular being remarkably constant in all systems.

Similar crystallite dimensions and organization have also been indicated for tooth dentine, but in tooth enamel the apatite crystallites were found to be not so markedly asymmetric, possibly variable in size, and certainly much larger than the ones in bone or dentine. This larger size of the crystallites in tooth enamel is clear from the wide-angle reflexions, which are much sharper than those from normal bone (Bale, Hodge & Warren, 1934).

All mature bone and dentine specimens seem to give rise to this characteristic low-angle X-ray scatter, and, in addition, some specimens of fish bone have been found to give a number of well-defined diffraction peaks (Finean & Engström, 1954; Carlström & Finean, 1954), which can be confidently associated with the organization of the apatite crystallites (Pl. 1). In the direction of the *c*-axis (i.e. parallel to the collagen fibre) there is a sharp and intense reflexion at 218 Å., and weak reflexions at about 650 and 130 Å. These reflexions could be accounted for as first-, third- and fifth-order reflexions of a fundamental repeating period of 650 Å. in the *c* direction, with a prominent subperiod of 218 Å. All these reflexions persist when the collagen has been removed from the system by refluxing with ethylene diamine for 48 hr. After this treatment the bone is brittle, it gives no organic residue on decalcifying with nitric acid, and it shows no carbonization on heating to high temperatures. In the direction at right angles to these sharp reflexions, the low-angle scatter corresponding to the diameters of the apatite particles shows a pronounced shoulder which disappears when the specimen is decollagenized. After removal of the collagen, the slope of the scatter in this direction can be readily interpreted in terms of a scattering particle of diameter 65–70 Å. If this scatter curve is subtracted from the original curve for the intact bone in the manner indicated in Pl. 1, a peak (curve 3) is obtained which, when treated as a Bragg reflexion, gives a spacing of 68 Å. On the face of it, this reflexion would seem to be associated with the collagen component, and in this respect it is interesting to note the renewed interest in the equatorial reflexions from collagen. On the other hand, the spacing agrees very well with the calculated diameter of the apatite particle, and it is conceivable that the removal of the collagen from the system could produce sufficient disorganization to destroy an already far from perfect spacing of the apatite crystallites.

All aspects of the X-ray diffraction data on bone are in keeping with a system of rod-shaped apatite crystallites of dimensions about 210 × 70 Å., and long rods of about the required diameter have been observed in electron micrographs of bone preparations (Finean & Engström, 1953), particularly of thin cross-sections of bone (unpublished data). However, in the electron micrographs crystal platelets have also been observed, and

these have been suggested to be the characteristic bone crystals. The ideal dimensions for these crystals suggested by Robinson & Watson (Robinson, 1952; Robinson & Watson, 1952) were 350 × 300 × 50 Å., but the X-ray diffraction data cannot be interpreted in terms of such a crystal, and the relationship between the rod-shaped crystallite deduced from X-ray data, and these additional platelets seen in the electron micrographs is not yet clear.

From these observations on the organization of the apatite crystallites it would seem that there is a significant relationship between the size and orientation of the apatite crystallites and the structure of the collagen fibres. The apatite particle length is one-third of the repeating period along the collagen chain, and in the best organized bone systems the fundamental repeating periods of the collagen fibres and the apatite structure coincide. There is little doubt that the collagen fibres control the crystallization of the apatite component either directly or indirectly through other substances attached to them at regular intervals. In this connexion it is worth noting that the unusual apatite organization in tooth enamel is probably to be associated with the fact that enamel does not contain collagen, but only a small amount of a keratin-like protein, whilst the high degree of order in some fish bones may arise from the exceptionally large amounts of collagen present, though there is also the possibility of an effect of the varying constitution of collagens which has been reported.

The observations emphasize the importance of structural repetitions at intervals of 210–220 Å. along the collagen chain. The existence of such prominent subperiods has been indicated from both electron microscope and X-ray diffraction data on collagen itself, though the fundamental period of the mature collagen is undoubtedly about 640 Å. This same fundamental period is in fact given by the fish-bone structure, though even in this case there would still seem to be particles of 218 Å., three of these constituting the full repeat. The question of exactly what in the collagen structure is active in promoting or perhaps halting crystallization in this regular manner is of great interest from the point of view of the structure of collagen itself. There does not seem to be any small repeat along the collagen chain which has any significance in relation to the 6·87 Å. *c*-axis dimension of the apatite unit cell, so probably the active centres are to be found at longer intervals. It is hoped to obtain more detailed information through *in vitro* studies of the deposition of apatite on modified collagens.

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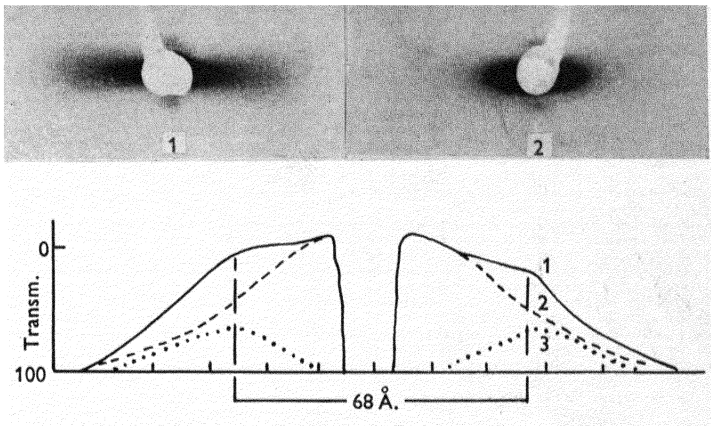

Low-angle X-ray diffraction patterns and photometer traces of intensities of low-angle scatter from a longitudinal section of pike membrane bone, before (1), and after (2), extraction of collagen with ethylene diamine. Curve 3 in photometer tracing diagram is obtained by subtracting curve 2 from curve 1.

(*Facing p.* 88)

FIBROGENESIS OF CONNECTIVE AND SKELETAL TISSUES IN THE EMBRYONIC FOWL

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PART I

A systematic investigation of the constituents in fibrogenic cells of the fowl embryo is being made in an attempt to shed light on the relationship of the cells to the mode of formation of the intercellular substance of the collagenous tissues. Cytoplasmic granules which contain polysaccharide protein components, ultramicroscopical structures, one of an apparently lamellar nature lying within the cytoplasm, and the other of fine filaments, have been studied by means of observations on the living material correlated with electron microscope and cytochemical techniques. Since these structures have been seen in fibrogenic cells both *in vivo* and *in vitro* it is possible that they may represent the precursors of the extracellular substance.

Ultramicroscopical elements

In a previous paper (Fitton Jackson, 1954*a*) a detailed account has been given of an ultramicroscopical organization observed under the electron microscope in thin sections of four types of fibrogenic cells situated in areas where the rapid formation of collagen is known to occur. Further investigation has shown, for example, that in sections through the tendons of the metatarsus the cytoplasm of the fibroblasts is seen to contain dense lamellar structures about 200 Å. in width. These elements often lie in pairs which are separated by distances of about 1000 Å. and are associated with, and in some cells are woven around, granules of less than 0·7 μ in diameter (Pl. 1). Similar observations have been made in osteoblasts, osteocytes and chondroblasts in six other types of collagenous tissues. It is possible that the intracytoplasmic lamellae observed in fibrogenic cells *in vivo* may be homologous with the ultramicroscopical elements that have been demonstrated in numerous other cell types (e.g. lamellae of Dalton (1951); chromidies of

* Parts I and II.
† Part II.

Bernard, Haguenan, Gautier & Oberling (1952); endoplasmic reticulum of Palade & Porter (1952); ergastoplasmic sacs of Weiss (1953); double membranes of Sjöstrand (1953) and Bradfield (1953)).

In addition, in sections from 11-day fowl embryos, fine filaments, about 100 Å. in diameter in cross-section, appear to arise in association with the cytoplasm, possibly at a certain depth beneath the somewhat ill-defined surface of the cell and thence to extend into the intercellular spaces: in some cells these filaments are seen to be in very close proximity to the nuclear membrane (Pl. 1). However, once these filaments are seen to be clearly extracellular, the individual units, or fibrils, as they may now be termed, are found to be packed into bundles: at 16 days' incubation the diameter has increased to about 250 Å. and a periodicity characteristic of collagen is often plainly visible, while individual fibrils are surrounded by a material of less density, which may be ground substance.

In an attempt to correlate these observations on the fixed and sectioned tissues *in vivo* with the appearance of the cytoplasm in whole cells, embryonic fowl tissues have been cultivated *in vitro* by the hanging-drop technique in a fibrin-free liquid culture medium. The cultures were grown on formvar-coated electron microscope grids and were fixed by buffered osmium tetroxide for from 30 to 60 sec. or by osmium vapour for up to 5 min., and subsequently washed in distilled water and dried. In fibroblasts from young cultures of tendon, and in osteoblasts migrating from explants of membrane bone, where there are as yet few if any submicroscopical intercellular fibrils present, the cytoplasm appears to contain a number of single dense filaments of varying length and about 200 Å. in diameter. Owing to the depth of focus of the electron microscope it is impossible to decide precisely in which plane of the cell the filaments are situated. In shadowed specimens, however, it can be seen that they do not lie on the upper surface, but whether they are within the cytoplasm or on the lower surface of the cell cannot be demonstrated by this technique.

It is possible, however, that the filaments seen in the cultures may be intracytoplasmic and that they may be identified with those described *in vivo*; though they are not so numerous in the tissue culture cells as in the embryonic tissue it must be remembered that the cells are not in the same environmental conditions and may not be so concerned in the production of extracellular material at this period. In tissue cultures observed after a slightly longer culture period the dense filaments that are associated with the cells appear to be either extruded or pulled away from the cytoplasm into the intercellular spaces by the movement of adjacent cells. A network arises of fine extracellular fibrils which at first show a regular periodicity of 70–80 Å. units, with a diameter of the fibril of about 200 Å. units. There

can be no doubt that once this intercellular mat has appeared further development of the individual fibrils of which it is composed occurs outside the cell; the fibrils increase in diameter while the periodicity differentiates through 210–640 Å. units.

Cytoplasmic granules

The presence of neutral red staining granules in osteocytes was observed by Dubreuil in 1910, by Fell (1932) in osteoblasts in tissue culture, and by many other workers. Gersh & Catchpole (1949) have postulated the presence in fibroblasts of barely resolvable intracellular granules of a glycoprotein nature, while Heller-Steinberg (1951) has suggested a similar component in frozen dried preparations of metaphysial bone cells of the rat, and associated the presence of these granules with the deposition of new bone.

Cytoplasmic granules of the order of 1 μ in size can be demonstrated in fibrogenic cells in histological and electron-microscope sections of suitably fixed material and in living cells either immediately after removal from the embryo or when cultivated *in vitro*. They are most easily studied in tissue cultures.

In living tissue cultures of frontal bone, metatarsal tendon and cornea, grown in serum exudate and viewed by phase contrast, cytoplasmic granules of from 0·7 to 2 μ in diameter are very conspicuous in the outgrowing cells; they are clustered around the nucleus and spread into the cell processes (Pl. 2, fig. 1). The behaviour of the granules has been recorded by time-lapse ciné-photography: they move very actively in the cell processes and are often orientated into lines of four to five granules and move independently to and fro in the axis of alignment. Under oil immersion the centre of the granules is seen to be less dense than the periphery and in some instances two or more granules appear to join together (within the limits of optical resolution). They persist throughout mitosis and are still plentiful when some intercellular material has been formed. Granules have never been seen to be extruded from the cells. On application of Janus green, the granules remain colourless whereas the filamentous mitochondria take up the stain. The granules are also stained supravitally by neutral red and gentian violet (Bast, 1921), which confirms the views of previous investigators.

Under the electron microscope osteoblasts, fibroblasts and osteocytes cultivated directly on electron-microscope grids and fixed by osmium tetroxide vapour show granules closely similar in appearance to those of the living cell; they are seen to have a diffuse margin and are less dense than the smaller sharply defined lipid globules (Pl. 2, fig. 2) that are also

present (Fitton Jackson, 1954*b*). Thin sections of cultures cut parallel to the surface of the migrating cells show variations of internal density in the granules; their central zone sometimes appears as a distinct region. In thin sections of tissues from the embryo the cytoplasmic granules in fibrogenic cells are found to be non-homogeneous in structure.

After fixation of the tissues by methanol, Zenker formol, osmium tetroxide or freeze sublimation the granules are well preserved and various cytochemical tests have been applied. Following treatment with periodic acid, Schiff's reagent will stain the granules bright red. This reaction can be obtained after extraction of lipid with methanol/chloroform at 50° C. for 12 hr., or after exposure to diastase. From this it may be concluded that the granules contain a polysaccharide other than glycogen. The presence of a free aldehyde group can be excluded, as the Schiff reagent stains the granules only after prior treatment with periodic acid. The polysaccharide may be acidic in nature, as a positive reaction is obtained by the dialysed iron procedure of Hale (1952) though this method may not be highly specific. It is of interest that even after prolonged treatment with periodic acid and Schiff's reagent only the periphery of the granules stain, while the centre remains colourless.

On addition of metachromatic stains (toluidine blue, thionine) to living tissue cultures, the granules acquire the red purple of metachromasia within 3 min. This may imply the presence of macromolecules with negative charges; that is to say, that the staining reaction could be due to sulphate, phosphate or carboxyl groups. It is important to stress that in cultures grown in Tyrode solution alone the outgrowing cells, from, for example, a tendon explant, also contain metachromatic granules; this would indicate that the metachromatic material has not been absorbed solely from the culture medium.

That the granules contain some protein can be demonstrated first by their ability to take up basic dyes, such as eosin, and secondly by ultra-violet microscopy of the living tissues where there is absorption at 280 mμ, but minimum at 313 mμ. There is also some absorption at 265 mμ; which could indicate the presence of a nucleoprotein. However, in preparations stained by the May Grunwald–Giemsa (Jacobson & Webb, 1952) and methyl-green pyronine methods for ribonucleoprotein and desoxyribonucleoprotein or by the Feulgen technique for DNP alone, the granules give no specific staining reaction for either nucleoprotein; nor are the granules destroyed by digestion with ribonuclease or desoxyribonuclease for periods sufficient to remove the known content of RNP or DNP from the cells. Thus it is an open question, on present evidence, whether the 265 mμ absorption are due to nucleoproteins which

the staining techniques have failed to demonstrate and the nucleases have failed to remove.

In cells growing in regions where extracellular material is developing the granules give an alkaline phosphatase reaction by the use of the Gomori–Tukamatsu technique, and this may well be correlated with the known phosphatase activity of fibrogenic tissue (e.g. Fell & Danielli, 1943). Application of the neatetragelium salt test of Shelton & Schneider (1952) suggests that dehydrogenase systems may be present in the granules.

PART II

Some preliminary attempts have been made to isolate the granules of fibrogenic cells for chemical investigation. Cell suspensions, consisting almost entirely of osteoblasts, were prepared by a modification of the culture techniques of Moscona (1952) and Rinaldini (1954) from the frontal bones of 11-day fowl embryos; the cells were grown in a fibrin-free liquid medium in bottle cultures. The cells were harvested on the fifth day, and homogenized in either 0·88 or 0·25 M-sucrose solution. The homogenates were fractionated by differential centrifugation following essentially the method of Hogeboom & Schneider (1950), and a fraction which contained particles was used in these studies. Such fractions, after washing by resuspension and re-centrifugation in sucrose solution, were shown by electron microscopy to be free from periodic fibrils typical of collagen, but the possibility that they were contaminated with either cellular components, such as mitochondria, or with non-structured extracellular material adhering to the particles cannot at present be excluded.

An interesting property of these isolated particles observed under the light microscope is their tendency to line up and form what seems to be a continuous long fibril with a regular beaded appearance. This transformation is aided by pressure on the cover-slip, by partial drying of the sucrose suspension, or by the addition of such stains as neutral red.

Preliminary chemical investigations indicate that these fractions are principally polysaccharide in nature, while the presence of hexosamine, revealed by two-dimensional paper chromatography of hydrolysates, indicates that the polysaccharide is, in part at least, mucopolysaccharide.

The fractions investigated so far have been shown to contain approximately 30% protein. Semi-quantitative amino-acid analyses by the method of Wellington (1954) indicate that mixtures of proteins are present. In one experiment a subfraction was obtained by extraction of the particle fraction with aqueous ethyl alcohol which gave an amino-acid analysis resembling that of collagen with large amounts of proline, hydroxyproline and glycine.

DISCUSSION ON PART I AND PART II

This work has demonstrated cytoplasmic components of fibrogenic cells, but it is too early to be certain of their precise nature and function. In conclusion, it is perhaps worth considering briefly their functional possibilities.

The dense lamellae are a consistent cytoplasmic feature of fibrogenic cells *in vivo*. Unfortunately their chemical nature cannot be demonstrated by cytochemical methods since they are well beyond the resolution of the light microscope, while there is no evidence on which to base a connexion between these structures and the known basophilic properties of the cytoplasm. Thus it must be borne in mind that although these lamellae may be concerned in chemical activities related to fibrogenesis, there may be no such connexion.

By electron microscopy a close correlation has been demonstrated in the development of the collagen fibrils *in vivo* and *in vitro*. When considering the possible mechanism of fibre formation, the lack of a well-defined cell surface would appear to be of importance, for there can be no doubt that the filaments seen in association with the cytoplasm are in fact immature collagen fibrils, but their precise site of origin and method of elaboration is not yet clear.

The cytoplasmic granules might function in various ways. By cytochemical methods they have been shown to contain polysaccharide and to have metachromatic properties, while chemical methods have indicated the presence of a hexosamine. These facts suggest that part at least of the polysaccharide is mucopolysaccharide. The granules contain some protein; as far as can be ascertained from observations on the intact cell, both protein and polysaccharide are present in the same granule.

Many observations by optical and cytochemical methods have shown that the granules are not homogeneous in structure and that they have a distinct central region. Enzyme activities may be localized within the granules, while the existence of still other components cannot at this stage be excluded; nor can the possibility be excluded that there may be some connexion between the filamentous lamellae and the granules.

The possibility that the cytoplasmic granules are merely storage granules for fibrogenic substances elaborated by other cytoplasmic components must be considered. The presence of phosphatase and of dehydrogenase systems within the granules suggests that these structures are capable of chemical synthesis and are concerned, perhaps together with other cytoplasmic components, with the production of polysaccharide and protein.

It is suggested that the polysaccharide and protein components of the granules may have fibrogenic properties; the granules may secrete, not necessarily continuously, a part or the whole of the ground substance; they may synthesize the collagen fibril itself or they may contain precursors for the complex extracellular material. The method whereby the polysaccharide and protein components leave the cell is not yet clear, however, for in the living cell no extrusion of intact granules has been recorded. It must be emphasized that whatever is the origin of the extracellular material, further development of the fibrils and the corresponding increase of the ground substance occur outside the cell. In this connexion it is of interest to recall that for many years it has been postulated that a cellular secretion may either itself precipitate out in the intercellular spaces to form the extracellular material, or it may interact physically or chemically with the intercellular medium to form the collagen complex.

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EXPLANATION OF PLATES

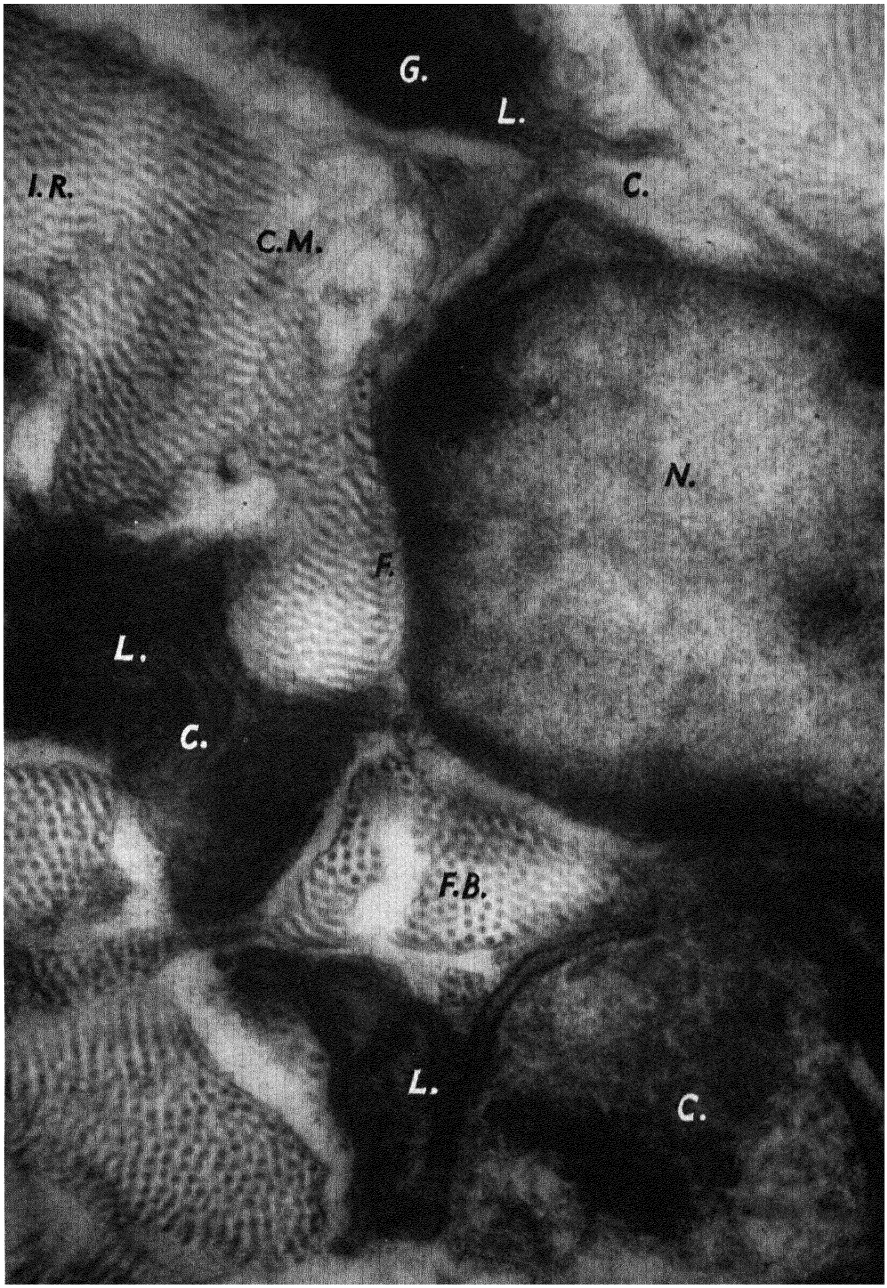
PLATE 1

Electron micrograph of a section through the tendon region in the metatarsus of a 16-day fowl embryo. Filaments (*F.*) are seen to be in close proximity to the membrane of the nucleus (*N.*), and extend into the intercellular regions (*I.R.*); no well-defined cell margin (*C.M.*) is visible. Dense lamellae (*L.*) are lying in the cytoplasmic regions (*C.*) while some are in association with a granule (*G.*). A fibre bundle (*F.B.*) is enclosed by cytoplasm, each fibril being surrounded by a less dense region, possibly the ground substance. Osmium fixation, methacrylate embedding. × 38,100.

PLATE 2

Fig. 1. Phase-contrast photomicrograph of an osteoblast growing from a living culture of frontal bone; the cytoplasmic granules are clustered around the nucleus. × 2500.

Fig. 2. Electron micrograph of part of the cytoplasm of a similar cell to that in Fig. 1. The diffuse periphery of the cytoplasmic granules and the more dense lipid globules may be seen. Fixed by osmium tetroxide vapour. × 8000.



For explanation see p. 96

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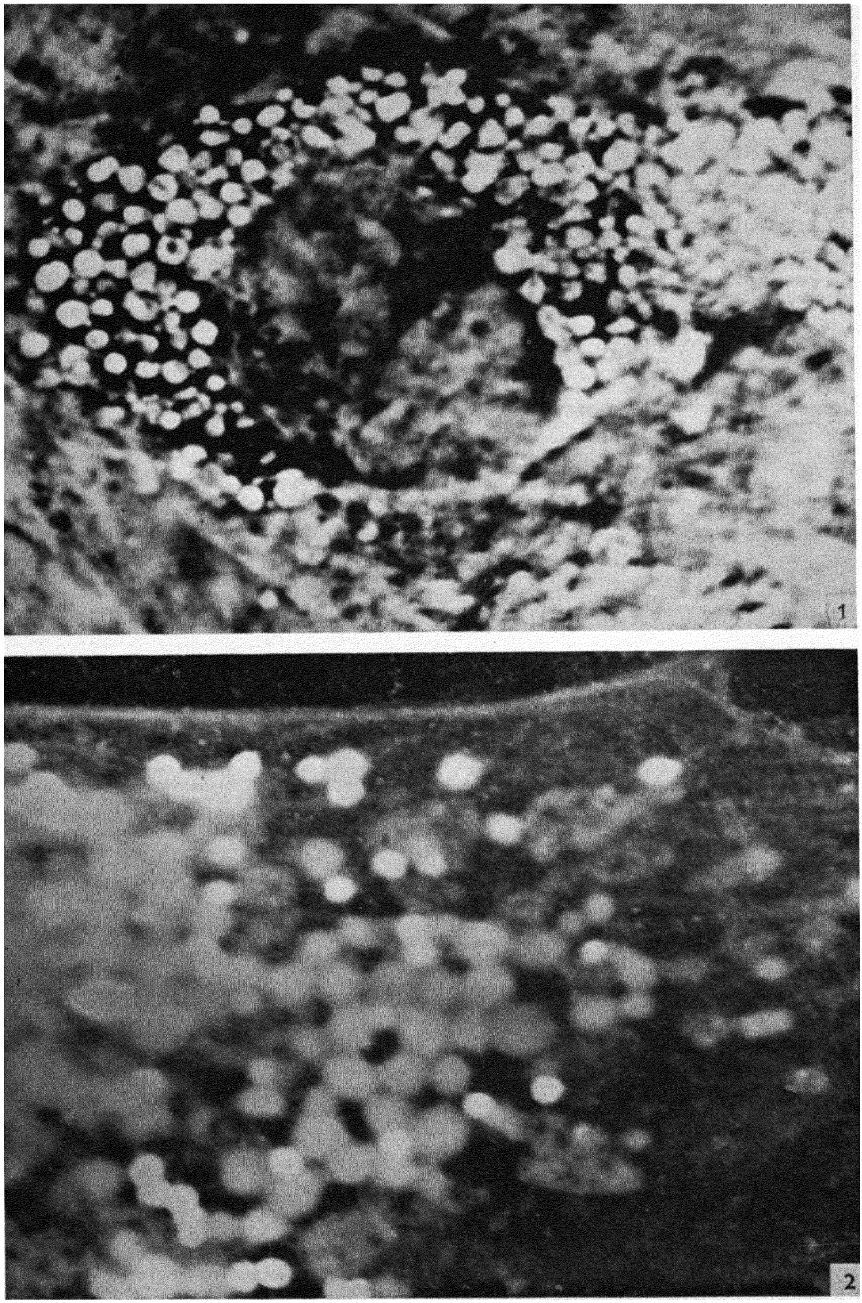

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CONFIGURATION OF COLLAGEN AND GELATIN MOLECULES IN CONDENSED AND DISPERSED STATES

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I. INTRODUCTION

In the spring of 1951 I prepared a review on 'The Structure of Collagen Fibrils' which presented a point of view based on pertinent physical and chemical data available at the time. So rapidly has the field been advancing since then that almost before the article was published in 1952 the point of view had begun to require some reorientation! I should like to use this occasion to discuss this reorientation in the light of selected newer developments, in particular reviewing results obtained recently by my students: Dr Carolyn Cohen, Dr Paul M. Gallop, Dr Malcolm A. Rougvie, Mr Harold W. Wyckoff and Mrs May-I Chow. Much of their contribution has not been published as yet and will have bearing on a number of the discussions of this Symposium.

Certain of the results of Cohen and Gallop only indirectly relate to the structure of solid states of collagen, so the present title has been broadened to include gelatin and dispersed states of collagen and gelatin. The identificational, distributional and many of the chemical aspects of the 1952 review will not, however, be reconsidered here.

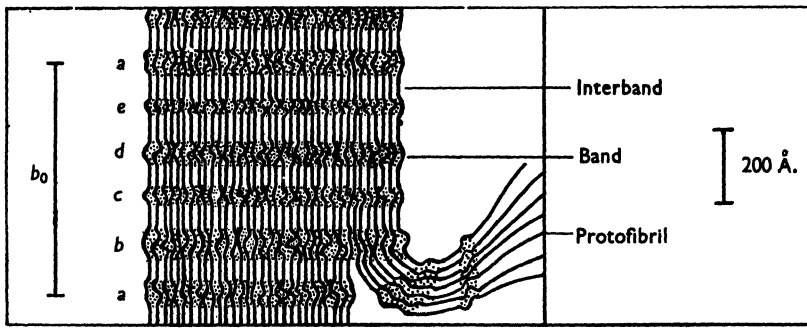
In order to conserve length I shall not attempt to cite, except where unavoidable, work of fundamental or historical importance appearing prior to 1951, considering that the earlier review or the more recent references can be sources for this sort of information. The subjects for discussion fall fairly naturally into the main headings given below, each of which can be treated more or less independently but has some bearing on the topics which follow it.

By way of introduction Text-figure 1 is given to illustrate diagrammatically the type of structure for the collagen fibril developed in the 1952 review, and to recall the meaning of certain terms which will arise again here.

This general picture for the fibril is still defensible relative to the types of detail shown, but the newer developments require some modification in finer features, in particular regarding the internal structure of the protofibrils, about which evidence was less compelling three years ago.

II. THE EFFECT OF TENSION ON X-RAY DIFFRACTION BY COLLAGEN

The demonstration by Cowan, North & Randall (1953) that the wide-angle diffraction pattern of dry collagen can be improved by maintaining tension on the fibre during X-radiation, has been widely hailed as making available additional and better information for structural analysis. Another important consequence is the fact that the better ordered parts of the fibril (interbands), believed responsible in large part for the wide-angle diagram, as well as the more poorly organized parts (bands), whose disorder was previously recognized at small-diffraction angles, are now both shown to be normally in somewhat relaxed states.



Text-fig. 1. The structural features of collagen fibrils accessible to electron optics and small-angle diffraction. The segment b_0 is to be repeated many times vertically, and details, such as the number and location of bands *a–e*, are only roughly indicated.

Rougvie & Bear (1953) have studied in detail the complex interplay of events, as disclosed at both wide and small angles, occurring when water is added to dry tendon (kangaroo tail). While they preferred to leave the tendon in its relaxed condition at each state of hydration, tension was noted to be of influence on the results at small angles. Tension produces, at small angles, effects in some respects similar to hydration of relaxed fibres: both influences straighten the intrafibrillar elements (protofibrils), as shown by increased macroperiod and the disappearance of the 'fanning' evidence for band disorder.

The effect of tension on diffraction by *dry* fibres at *wide* angles is shown in Pl. 1, fig. 1*a*. Fig. 1*b* indicates the comparable result obtained by Cohen (1954) with a *moist* specimen, which was also held under tension during exposure to X-radiation. This diagram is of importance because it has lost nearly all evidence of sharp row-line structure, for reasons which will appear below in § IV.

The wide-angle diagrams obtained with Cu $K\alpha$ radiation from stretched specimens show, among other changes which have been discussed by others, a decrease in the intensity of the 3·0Å. meridional arc. This occurs because the reflected intensity is pulled closer to the fibre axis in reciprocal space and is thus removed from the sphere of reflexion under normal conditions of incidence of the X-ray beam. Pl. 1, fig. 1*c* shows the result of avoiding this difficulty by using shorter wave-length radiation (Mo $K\alpha$) with dry, stretched tendon. The 3·0Å. reflexion is then seen to remain, next to the principal equatorial arc, the strongest diffraction of the wide-angle diagram.

III. THE DENSITY OF COLLAGEN

In any discussion of models for the structure of collagen fibrils the consideration of density is important in evaluating the correctness of the space filling achieved. It is well known, however, that densities appropriate to the structure of fibres as determined by X-ray methods ('micellar' densities) may be 5–10% higher than observable macroscopic ones. The problem is further complicated in the case of collagen because of manifold indications that the density varies appreciably at different locations along the fibril. The use of density as a measure of 'average' condition, however, prevents one from departing too far from reasonable conclusions.

Pomeroy & Mitton (1951) have carried out what is probably the most reliable examination of collagen (dry calf-hide) density. They found, by a method involving displacement of nitrogen gas, a 'real density' of 1·41. By displacement of non-polar solvents the results were about 5% lower (1·34). Other measurements by gas displacement on dry leathers or hide powder have given results as low as 1·22 (Spiers, 1952) and as high as 1·6–1·7 (D'Orsi, 1952). Pomeroy & Mitton, as well as Spiers, have found also that collagen densities are appreciably increased by the addition of strongly bound moisture, suggesting that much of this water disappears within the collagen structure. On the other hand, Rougvie & Bear (1953) found that the water strongly sorbed by kangaroo tendon at low humidities is apparent as the proper amount of extra volume according to the observed X-ray spacings. The means of reconciliation of these apparently contradictory observations is not clear, though it is possible that the complex tissue structure of a leather may not provide the best place to study fibrillar or subfibrillar properties.

For purposes of fine-structure analysis it is desirable that the X-ray and density data be available for a fairly easily reproducible standard state of the same collagen preparation. These conditions are thus far met only in the case of the kangaroo tendon studied by Rougvie & Bear. Thoroughly

dried, compact, transparent pieces of fibre yielded 1·34 as density, by flotation in mixtures of bromoheptane and carbon tetrachloride or chloroform, and with X-rays the following spacings: macroperiod near 600 Å., wide-angle meridional spacing 2·86 Å. and equatorial spacing 10·6 Å. For reasons cited above the true X-ray density may be possibly about 5% higher, around 1·41 Å.

Note that the standard or relaxed state just used for density consideration is different from that described above as the simple, extended condition. The two represent extremes of the situations normally met with in X-ray diffraction study. The relaxed state is to be used when space-filling features of models are to be examined in terms of average interfibrillar density and spacings. The extended state represents a useful condition in which the disorder of the fibrils has been reduced. Density for the latter condition is more difficult to measure.

For purposes of §V below, it is useful to calculate the mass per unit length (M/L) of a protofibril in the dry relaxed fibril. With pseudo-hexagonal packing, the protofibrillar axes are 10·6/cos 30° or 12·2 Å. apart. Each protofibril has cross-section of 127 Å.^{2}, which at a density of 1·41 corresponds to a M/L value of 108 avograms/Å. Extension of the fibrillar macroperiod from 600 to 700 Å. would decrease M/L to about 93. Unrestrained fibres in water take on macroperiods of about 660 Å., corresponding to $M/L = 98$. Calculations of this sort assume that the cross-sectional area of the protofibril is everywhere that appropriate to the interbands believed to yield the equatorial arc (10·6 Å. in the relaxed standard).

Justification for this approximation is found in the two indications of electron-optical studies: (*a*) that frayed fibrils apparently contain equal numbers of filaments, hence probably also of protofibrils, running through both band and interband cross-sections, and (*b*) fibrillar cross-sections at bands and interbands are not markedly different, as they would be if protofibrillar diameters varied greatly between these two types of location.

When examined electron optically in detail fibrils do, of course, differ in diameter at bands and interbands, even when remaining in the 600–700 Å. macroperiod range accessible to X-ray study. Corresponding density differences also occur, and as Hofmann, Nemetschek & Grassmann (1952) point out, the greatest fibrillar densities (with respect to electron transmission in the case of electron-optical investigations) are found at bands, where Text-fig. 1 shows greatest protofibrillar disorder. It is not correct to suppose, however, as these authors do, that disorder must be associated with decreased density, but only that the same local constituents will have less density than they would if well ordered. The density at any locality depends on both order (or packing) and the atoms present. It is perfectly

possible for interbands to remain lighter than bands, in spite of better packing in the former, particularly if the intrinsically less dense residues are concentrated there (see Table 6 and associated discussion of Bear, 1952).

IV. HELICAL POLYPEPTIDE CHAINS IN COLLAGEN

Although up to and including 1951 there had been a number of indirect reasons which led investigators to propose helical models for the configuration of the polypeptide chains in collagen, it was not until Cochran, Crick & Vand (1952) had worked out the Fourier transforms for helical structures that it became possible to examine wide-angle patterns directly for the existence of helical configuration. Immediately a number of laboratories recognized independently that the collagen diagram does indeed bear characteristics of helical structure. It also became apparent that all of the earlier models were in error (Pauling & Corey, 1953).

Cohen & Bear (1953) were able to derive certain specifications required of the collagen helix from the diagrams of normal (relaxed) type, and concluded that a discontinuous helix consisting of seven roughly equivalent scattering groups of atoms in two turns along 20 Å. of axial projection was indicated by the positions of major diffractions. The fibril would contain these helical elements (protofibrils) in pseudo-hexagonal packing, with about 12 Å. between helix axes in dry fibres, to account for the principal equatorial reflexion.

A helical structure need not have a finite axial periodicity, but it is often possible to discuss it with a sufficient degree of approximation to the truth by choosing the smallest axial periodicity which agrees with the observed diffraction layer-line positions within experimental error. This situation is well illustrated in the collagen case: Cohen & Bear initially felt that a two-turn, 20 Å. period was reasonably satisfactory, but it developed that slightly improved accuracy attended adoption of a three-turn period. Investigators in the field are, I believe, now fairly well agreed that the collagen structure is to be described in terms of the following specifications: there are ten nearly equivalent scattering groups distributed on three turns of a *genetic* helix. This three-turn unit occupies 28·6 Å. of axial projection in the case of relaxed material, or close to 30 Å. of projection in the extended condition.

The meaning of 'equivalent scattering group' in the above description requires further consideration. In most helical protein structures which have been under discussion this group consists of the atoms of a *single* residue, but density shows that this cannot be the case in collagen. By reference to the M/L values estimated in § III, it is readily seen that a protofibrillar segment of length 28·6 Å. would contain mass of 3090

avograms, enough for 33 residues of average weight 93. Since there are ten groups along 28·6 Å. of helix length, each group must comprise approximately three residues. There could be as many as four residues per group if less than average-sized residues are considered to be at the interband segments whose structure is presumed to be under analysis.

The use of the word 'genetic' in the helix specifications also is of some importance. It is borrowed from a similar usage in botany to describe the arrangement of leaves or bracts on plant stems (phyllotaxis, see Thompson, 1942). In the X-ray analysis, each equivalent group of scattering atoms is analogous to a leaf or bract, but since the X-ray analysis involves polypeptide molecular chains there is added the problem of how the scattering groups, more specifically the amino-acid residues comprising them, are chemically connected. Information regarding the chemical connexion does not come as simply from the X-ray diagram, which furnishes most immediately the relative distribution in space of the centres of the nearly equivalent groups.

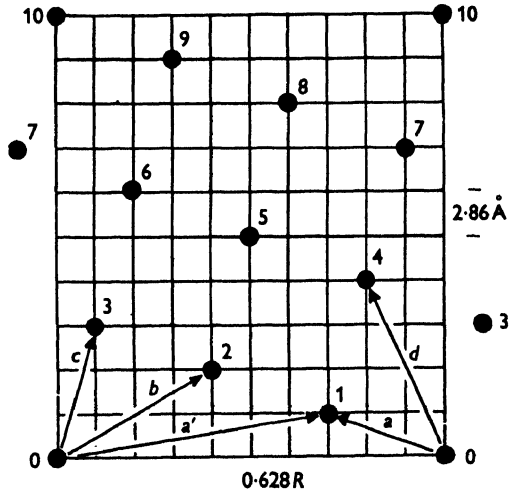
Again borrowing from diagrams found useful in the study of phyllotaxis, one finds consideration of the problem of chemical connexion in helical polypeptide structures facilitated by construction of the helix-net shown in Text-fig. 2. The nodes of the net represent locations of centres of equivalent X-ray scattering power, and they are distributed over an unrolled cylindrical shell originating about the helix axis. Horizontal coordinates are proportional to the shell radius, which ideally is to be measured from the separation of intensity maxima on the layer lines from the meridian of the diffraction pattern.

What may be called 'connexion ambiguities' in the possible structures arise from the fact that various ways of connecting nodes, as well as residues within nodes, all correspond to helical structures which do not violate the essential nodal distribution. If the manner of connexion requires only one helix running through all nodes, the model is said to be 'primitive'. Of the two possible primitive helices, with three or seven turns per axial period in the collagen case, the one with the fewer number of turns is called 'genetic'. Although cable models are also possible, the statement of helix specifications in terms of the genetic arrangement of nodes provides the simplest way to state the requirements dictated by the positional features of the diffraction effects.

Another type of difficulty, the 'net ambiguity', arises from the theory of diffraction by helices. If the simple helix net, instead of being 1 net-cell wide as shown in Text-fig. 2, is 2, 3, or 4, etc., cells wide, but at the same time the nodal radius is approximately doubled, tripled, or quadrupled, etc., one comes again to nodal distributions which satisfy the positional

aspects of diffraction. With each of the possible nets additional connexion ambiguities arise, and, further, unless density requires that each node of a given net can contain only one residue, one must entertain the possibility that cables may be made of individual helices constructed on different nets, at different radii.

I have described the above ambiguities in some detail to make it clear that, while the application of transform theory has limited the helical models which may be entertained for collagen, the restrictions are far from capable of isolating a unique structure. Investigators active in this field realize



Text-fig. 2. The helix-net derived from wide-angle diagrams of collagen. Black dots represent the relative locations of centres (nodes) of equivalent groups of atoms on a cylindrical shell of radius R about a vertical axis. The vectors **a** to **d** show several possibilities for connecting nodes by means of polypeptide chains to form helical structures. Of the primitive connexions **a** and **a'**, the former is genetic. The axial projection per node (2·86 Å.) can be increased by applying longitudinal tension to the diffracting fibre.

these difficulties. For example, the model recently proposed by Crick (1954) is built on the double-cell net, instead of the simplest single-cell net, and Cowan *et al.* (1953) mention various possibilities involving both connexion and net ambiguities, although in their description of these the word 'residue' should rather be 'node', since variable numbers of residues will be assigned to each node, depending on density considerations. Density also limits the number of cells of width (a maximum of 4 in collagen) that need to be considered in the net ambiguity, since each node must have available at least one residue.

The possibilities are numerous, and no systematic coverage of them has appeared as yet. Fortunately, knowledge of stereochemical properties of polypeptide chains is now considerable and can be used to discard many

of the formally apparent structures. Miss Cohen and I hope to complete a systematic attempt in this direction before long.

Possibly the most formidable difficulty facing this task is caused by lack of knowledge concerning the role to be played by the pyrrolidine residues, so prevalent in collagen (about 22% of the residues) and whose atomic configuration is uncertain at crucial points. Some details are available from crystal structure determinations of L-hydroxyproline (Donohue & Trueblood, 1952) and of the copper salt of DL-proline (Mathieson & Welsh, 1952), but knowledge of the imide bond configuration in peptides involving the pyrrolidines is still lacking. Although there is biochemical evidence of a strong tendency for proline and hydroxyproline to be connected with glycine, the most definitive biochemical investigations yet available (Schroeder, Kay, LeGette, Honnen & Green, 1954; Kroner, Tabroff & McGarr, 1953) tell little about the way in which residues, such as the pyrrolidines, are to be distributed along the polypeptide chains. Further, since there is reason to believe that the wide-angle diagram does not result from every portion of the collagen fibril, one does not know how much of the available proline or hydroxyproline to include in the helical structure derived therefrom.

The relative merits of the wide-angle patterns from dry and wet collagen in their use for the structural analysis are worth some consideration. While the dry condition yields a diagram more useful in terms of number and sharpness of layer lines and applicable to estimation of the separation between individual helical elements in the fibril (from measurement of the prominent 10–11 Å. row line), it is, on the other hand, the diffraction effects of these interactions between *neighbouring* helices that reduces value for studying details of the structure of *individual* helices. The pattern of moist material (Pl. 1, fig. 1*b*) is more satisfactory for this latter purpose, since it shows many aspects expected of single helical structures, i.e. the diagram is roughly the Fourier transform of a single discontinuous helix or cable.

Apparently hydration has caused individual helices to scatter X-rays largely independently. Study of the positions of maxima and minima of intensity on the equatorial and third-layer lines of the diagram of moist collagen, examined in relation to the properties of appropriate squared Bessel functions, indicates that the collagen helix has major density of scattering atoms on a shell of radius about 3 Å. if analysed according to the single-cell net. This would mean approximately twice or three times this radius if double or triple cells are considered. Since the separation between helix axes in the dry condition is as small as 12 Å., it seems doubtful that the major components of the structure could be built on three-cell or higher nets.

The ultimate testing of helical models, when they have reached the stage of formulation in terms of detailed atomic co-ordinates, is most easily done by means of optical diffraction apparatus, similar to that now in use by many diffractionists. In our laboratory, Mr Wyckoff and Mrs Chow have developed a practice in which a drawing is first made of a projection of one period of the atoms on a plane through the helix axis. This is once reduced in scale photographically to a size suitable for compounding a longer helix by combination of several periods in series. A final photographic reduction yields a 'mask' of scale about 0·14 mm. = 1 Å., which is held with immersion oil between two optical flats to serve as an aperture for diffraction 'antipoint' registration, in a system which uses the Hg green line with lenses of long focal length (2 and 4 m.).

While details will be published elsewhere, an example is given in Text-fig. 2, which examines the detailed model proposed for collagen by Crick, consisting of two chains wound about a common diad axis. Each chain has ten pairs of residues in 1·5 turns along the period of 29·5 Å. Residues within each of the two sets of alternating residues are equivalent and the members of one set can be pyrrolidines. Wyckoff and Chow have prepared optical transforms for the two-chain skeleton of amide groups, for the skeleton plus β-carbon atoms on all residues, and, finally, for a structure with maximum (excessive) content of proline side-chains added. The optical transforms for these structures, arranged in the order given, become progressively similar qualitatively to the actual collagen X-ray diffraction pattern. The final result is shown in Text-fig. 2, from which it becomes apparent that the model fails in one important respect: the intensity maxima on the third and seventh layer lines are too widely separated from the pattern meridian, corresponding to the fact that Crick's model must contain too many radial atomic vectors which are improperly small for a structure based on the double-cell net.

Crick's model also suffers from the objectionable feature that it is composed entirely of *cis*-amide groups; Corey & Pauling (1953) prefer *trans*-amide groups for polypeptide chains. Also, a number of interatomic contacts are small, as Crick himself has noted. It has also been reported that the results of infra-red dichroism studies are not in agreement with the proposed model (Badger & Pullin, 1954; Sutherland, Tanner & Wood, 1954). Indeed, Crick now agrees that the success of his model is questionable. It was, however, the first detailed structure which could account for major features of wide-angle diagram, as well as for reasonable density.

The new model suggested by Huggins (1954) is based on a single-cell net, with three residues per node, of which one is a pyrrolidine and another glycine, leaving the third to be other than these. Two of the three peptide

bonds are of *cis* rather than the preferred *trans* type, and close-packed arrangement of the helices yields a density of 1·30 for the anhydrous structure (1·38 if one water molecule is added per three residues). The anhydrous density, already rather low, is based on an average residue weight of 93, and would be still less if residues of smaller average weight were, as is suspected, present at the interbands. The model also does not seem well adapted to the requirements of infra-red dichroism. Optical transforms have not yet been prepared for this structure.

Ramachandran & Kartha (1954) have recently made very interesting proposals for the structure of collagen in which are used hexagonal cells: $a = 13{\cdot}4$, $c = 9{\cdot}55$ Å. for the air-dried condition and $a = 15{\cdot}0$, $c = 9{\cdot}20$ Å. for the moist state. The cells have a symmetry of space group $P3_1$, with nine residues per cell arranged in three independent helical chains clustered closely about the three-fold screw axes on cell edges parallel to c. Each helix has three residues comprising one helical turn along 9·55 Å. The prominent meridional arc at 2·86 Å. cannot be assigned true diatropic character but is believed to be a poorly oriented, near-diatropic reflexion (113). Outside of the fact that this analysis of the collagen wide-angle diagram represents a rather forced and complex interpretation of a situation which helix transform theory, not applied here, can more simply explain, the recent diagrams of stretched material rather emphasize that the 2·86–3·0 Å. reflexion is truly diatropic.

It would appear, therefore, that while helical polypeptide chain configuration seems definitely indicated for collagen, no detailed model can be recommended as yet. It seems likely that a successful conclusion to this problem may be forthcoming soon, provided difficulties connected with the role of the pyrrolidines can be circumvented.

V. PROTOFIBRILLAR DISPERSIONS OF COLLAGEN

Although acid dispersions of collagenous particles, capable of reconstitution into fibrous products, have been known for some time, only recently has evidence begun to accumulate relative to the state of the dispersed particles. An account of such work since 1951 begins with the study of M'Ewen & Pratt (1953), who used light scattering and viscosity to investigate citrate extracts of rat skin as well as rat-tail tendon collagen dissolved in acetic and hydrochloric acids. Gallop (1955) has performed similar studies on citrate solutions of a previously 'recrystallized' product obtained by citrate extraction of carp swim bladder (ichthyocol).

The results of these two investigations are in reasonably good agreement, considering the difficulties inherent in the methods and the variety of

sources and conditions used in preparing the solutions. Particle weights (M) are found to occur in the range $1{\cdot}7$–24×10^6, and although the particles are apparently polydisperse, appropriate calculations reveal them to be thin (11–16Å.), randomly supercoiled particles of great contour length ($L = 13{,}400$–$160{,}000$Å.). Of particular interest are the M/L values, which lie in the range 54–120 avograms/Å., indicating that these filamentous particles carry, on the average, about one amino-acid residue per ångström unit of length. Gallop also estimated M/L from sedimentation (which for long particles of this sort is more nearly a function of segmental cross-section than of actual total size), obtaining a value of 88 avograms/Å., in reasonable agreement with the light-scattering figures.

From these results it seems difficult to escape the conclusion that the acid-dispersed particles of collagen are, in fact, essentially the protofibrils which have been postulated from X-ray diffraction and electron-optical studies of normal collagen fibrils. As calculated in §III above, from density and X-ray considerations of the normal solid state, M/L values of protofibrils should be 93–108 avograms/Å. The dispersed protofibrillar particles have contour lengths which are several to many times the macroperiods observed for all currently recognized fibrous varieties of collagen. If the protofibrils are freed into solution without far-reaching changes in the value of M/L, it is likely that they retain within their supercoiled segments the helical configuration which the diffraction analysis indicates is normally present.

Another line of evidence furnishing some light on this possibility comes from optical activity studies. Cohen (1954) has measured the specific optical rotation in solutions of Gallop's citrate-extracted ichthyocol and a similar calf-hide preparation, including the wave-length dependence. She finds that $[\alpha]_D$ for both is about $-350°$, hence more nearly comparable to Robinson's (1953) 'cold' gelatin value ($-262°$) than to his figure for 'hot' gelatin ($-128°$). Similar relations are demonstrable in the dispersion data.

It has been known for some time that films prepared from hot gelatin solutions are found to be amorphous when examined with X-rays, while films prepared from cold solutions show the wide-angle diffraction pattern characteristic of collagen, now understood to be indicative of helical polypeptide configuration. Robinson also found that solid collagen and films from cold-evaporated gelatin have the same infra-red NH stretching frequency (3300 cm.$^{-1}$), which is altered in the hot-evaporated films (3310 cm.$^{-1}$). He suggests that the cold gelatin forms possess a specific 'collagen fold', whereas the hot gelatin does not.

Cohen now adds the hypothesis that the specific fold is actually helical, and that this helical fold is more extensively preserved in cold acid solutions

of collagen than in cold gelatin solutions, as shown by the higher (negative) rotation of the former. A helix furnishes one of the classical examples of an enantiomorphic structure, and helical polypeptide chains may be expected to show rotation beyond that ascribable to their asymmetric carbon atoms alone. The high negative rotation of collagen is indeed outstanding among those of native proteins, which generally lie in the range -30 to $-60°$. Cohen suggests that the prevalent pyrrolidine residues, in introducing rather restrictive imide connexions to other residues, impress upon collagen polypeptide chains the one sense of helical twist consistent with the stereochemical configuration of their Cα atoms. In other native proteins large cancelling effects may occur because of almost equal stability of helices with opposite twists, which become possible (or even, perhaps, promoted) when relatively few pyrrolidines are present.

Gallop and Cohen have found that above about 30° C. ichthyocol in citrate buffer (pH 3·7) in a matter of hours or minutes (depending on temperature) loses intrinsic viscosity, which falls from about 13 eventually to 0·3 to 0·5, as well as specific rotation, which changes, even more rapidly in some cases, from about $-350°$ to near $-110°$. (Similar events take place in calf-hide extracts, but at somewhat slower rates.) Gallop (1955) has characterized the resulting product by means of viscosity, sedimentation diffusion and light scattering, finding an apparently monodisperse population of particles of weight close to 70,000, axial ratio 23 to 18 depending on hydration assumptions (0–30%). Since this mono-disperse product is obtained under conditions capable of breaking only weak, heat-labile bonds, Gallop retains for convenience the term 'parent gelatin' proposed by Scatchard, Oncley, Williams & Brown (1944) under similar circumstances.

Cohen notes that the rotation of the warm parent gelatin falls in the range found for other 'denatured' proteins (-80 to $-120°$). This would be understandable if in all of these cases the optical effects of specific folds or coils have been lost, with only variations due to compositional differences remaining.

A simple calculation reveals that the original protofibrillar ichthyocol particles would contain weight of 70,000 in 583–795 Å. of contour segment, the variation resulting from the range of M/L values (88–120) observed. The close correspondence of this segmental length range to that of the macroperiod (600–700 Å.), characteristic of normal collagen fibrils, gives rise immediately to the suggestion that the thermal change observed in going from the protofibrillar ('native') condition to the gelatin ('denatured') condition involves the breaking of weak bonds periodically linking monomeric units of weight 70,000 end to end. According to X-ray specifications, this unit would have an initial axial ratio of 35 to 58, so that the gelatiniza-

tion process, in reducing this to about 20, introduces considerable randomness, as is also indicated by the decline in optical activity.

The postulated collagen unit is, of course, a hypothetical particle and has not been isolated with certainty to date, although claims to this effect have been made twice. The first was that of Bresler, Finogenov & Frenkel (1950), who described a 'procollagen' particle of weight 70,000 in citrate extracts from ox skin. More recently Mathews, Kulonen & Dorfman (1954), working with citrate extracts of rabbit skin, reported a particle weight of 74,000 as measured by osmotic pressure, but their light-scattering results with the same system yielded weights of the order of 10^7. Their reported intrinsic viscosity (15) was also high. Thus, the light-scattering and viscosity results are quite at variance with those from osmotic pressure. Since they performed the latter at 30° C. and permitted lengthy equilibration at this temperature, it seems possible that their measurements were on a system in which appreciable conversion to parent gelatin had occurred. Osmotic experiments yield number average weights, hence are particularly sensitive to the presence of smaller particles. Indeed, one may possibly conclude that Mathews *et al.* have, in fact, produced additional evidence for the monodispersity of parent gelatin particles, since their figure for number average weight is close to the quite different weight averages provided by sedimentation-diffusion and light-scattering studies of distinctly gelatinized preparations. If their interpretation should be supported, however, they will have provided evidence for the independent existence, in the dispersed state, of monomeric particles of collagen (as distinguished from gelatin), having weight *c.* 70,000.

In our experience the single property which provides the best test as to how far a dispersion of native collagen particles may have progressed toward conversion to parent gelatin is provided by measurement of the optical rotation. This is capable of indicating directly the amount of matter transformed and does not depend on such difficult things as number or weight averages of the particles converted. Unfortunately, neither Bresler *et al.* nor Mathews *et al.* provide data on optical activity.

In all of the physico-chemical studies of collagen solutions, and the gelatins derived therefrom, one is never dealing at one time with the whole spectrum of collagen fractions. The question naturally arises as to the general validity of concepts regarding monomeric or protofibrillar particles, conversion to monodisperse parent gelatin, etc., derived from studies of the few fractions which have been examined as yet. It is, of course, impossible to feel wholly reassured on such points, but the following considerations indicate the usefulness of single-fraction studies. For example, the 'recrystallized' fraction of citrate-extracted ichthyocol, studied

by Gallop and Cohen, can be shown to be reasonably pure with respect to carbohydrate (0·005–0·006 g. of hexose per gram of dry ichthyocol) and foreign protein (ultra-violet absorption at wave-lengths longer than 2500 Å. is low). The solid yields typical wide-angle diffraction of collagen and, although indications of the normal 640 Å. period at small diffraction angles were faint, clear evidence of this structure in high yield was found electron optically. Furthermore, it is also possible to prepare from this material, the long-spacing (LS) modifications of Schmitt, Gross & Highberger (1953), who, however, have done much of their work with mildly alkaline phosphate extracts. In all of these ways, then, the citrate-extracted fraction of ichthyocol seems typical, and it is possible to demonstrate essentially complete conversion of this fraction to monodisperse parent gelatin of weight 70,000.

Experiments of this sort provide some degree of confidence that study of fractions does provide significant information regarding collagens generally, at least in relation to the structural considerations involved in the present discussion. They also indicate that the differences which exist between fractions probably reside in more obscure factors, such as the constitution with respect to side-chains, the presence of naturally occurring 'impurities', the possible existence of occasional interprotofibrillar cross links, etc.

VI. RECONSTITUTION OF FIBROUS MODIFICATIONS

An outstanding difference between the protofibrillar dispersions of native collagens and solutions of parent gelatins is to be noted in the fact that only the true collagen preparations are capable of yielding reconstituted fibrous or segmental forms exhibiting distinct macroperiods with characteristic intraperiod band patterns. Three such modifications are now known: the normal form, with average macroperiod of about 640 Å., and two others with much longer spacings (LS varieties) in the range 1500–3000 Å. (Schmitt *et al.* 1953).

The developments in this area are well covered by other papers in this Symposium, and at this point need be mentioned only the aspects of this work that relate to size, shape and configuration of the elementary particles which may be conceived as taking part in the formation of these ordered systems. Gross, Highberger & Schmitt (1954) have convincingly demonstrated that all three forms are essentially completely interconvertible under appropriate conditions, hence that they may be safely assumed to be different manifestations of the ordered arrangements possible for the basic elementary type (or types) of particle characteristic of collagen.

By study of certain illuminating manifestations, such as segmental breaks or dislocations at period boundaries, the time sequence of events occurring during formation, etc., Gross *et al.* have provided strong evidence that the LS forms are the product of side-to-side aggregation, with ends more or less in register, of long thin particles of length approximating the magnitude of the macroperiods. These elementary particles they name 'tropocollagen'. The protofibrillar particles present in collagen solutions are tentatively presumed to be end-to-end polymers of tropocollagen.

Since the LS forms are relatively new, there are as yet lacking many facts, such as density, optical rotation, composition relative to additives, etc., which would be useful in attempting to consider details of how the tropocollagen particles fit into these structures. Two significant features may be mentioned, however. Gross *et al.* report that the LS forms yield wide-angle X-ray diffraction recognizably similar to that of standard forms of collagen and gelatin. This observation indicates that, at least along an appreciable fraction of their lengths, the tropocollagen particles possess the same type of helical polypeptide configuration present in the original protofibril from which they were derived. It is also said that the variability of LS macroperiods, as observed electron optically, is nearly as great as found with normal collagen. This indicates that tropocollagen particles are probably markedly extensible, again possessing a property in common with protofibrils.

It is apparent from the above descriptions that the two elementary particles, the collagen unit of weight 70,000 and the longer tropocollagen particle of unknown weight, have some similar properties because of presumptive common protofibrillar origin. Since both unitary elements are hypothetical at present and have not been isolated for direct characterization, it has been difficult to discover which (if, indeed, either) may be more fundamental, or what is their relationship.

At present the outstandingly apparent difference is length; the 70,000 unit is thought of as 600–700 Å. long in normal collagen fibrils, and the tropocollagen particle as having length in the range 1500–3000 Å. when built into the LS forms. Two simple possibilities exist: either (*a*) both are different manifestations of the same elementary unit, with the shorter particle extended several fold when it becomes the longer one, or (*b*) the longer form is a polymerized product of the shorter one. In the 1952 review I inclined towards explanation (*a*), but it has gradually become apparent that LS varieties, hence the tropocollagen particles, are considerably variable in length, hence probably also extensible, and that full extension of a 600–700 Å. unit would not readily account for modifications as long as 3000 Å., particularly when wide-angle diffraction remains

unchanged, indicating that at least some of the normally coiled molecular chains have not been unrolled. Nevertheless, the possibility of some degree of unfolding exists, and one cannot conclude directly from a comparison of lengths the degree of polymerization, relative to the shorter units, which might exist in the tropocollagen particles. Possibly both stretching (*a*) and polymerization (*b*) are involved.

A few remarks about the phenomenon of extensibility are in order, since I laid considerable emphasis on this in the 1952 review. Unless it should develop that the marked extensibility of normal and LS varieties of collagen, as seen electron optically, is largely the result of electron bombardment or other artificial circumstance, the observation in all cases of at least two-fold variation in macroperiods without loss of characteristic intra-period band structure would establish the phenomenon's reality and importance. It may not be necessary, however, to explain this property in terms of the stretching of tightly wound, primitive helical coils, as was proposed earlier. For example, if helical cable models should turn out to account for the wide-angle X-ray diagram, they will be incapable of great local extension, but, if formed from twisted loops of some size, these might be pulled out during large extension.

Some protofibrillar extensibility resides in the disordered localities (bands) without influence on the local regions (interbands) responsible for wide-angle diffraction. One can realize observation of only limited (*c.* 10%) extension of interbands, with retention of the normal helical structure, when employing X-ray diffraction. Electron optically, however, great extension of interbands can be observed under these special conditions (Hofmann, Nemetschek & Grassmann, 1952). Until the effects of these conditions are better understood, it seems unwarranted to maintain the requirement of marked extensibility as a criterion for model building in relation to phenomena covered by wide-angle diffraction.

VII. CONCLUSION

The discussion above clearly indicates that facts about collagen structure have become available in increasing significance since 1951. This has meant a considerable restriction in the freedom with which working hypotheses are constructed, but in turn conceptual frameworks can become more and more tightly connected with reality. Some of the recent developments are already so close to crucial relationships that one feels particularly optimistic that much will be decided in the very near future. It may soon be true, if not already, that more is known about the structure of collagen than about any other fibrous protein.

I wish to acknowledge with particular pleasure the enthusiastic and productive contribution to many of the developments described above provided by my student colleagues mentioned in the introduction. This work was supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and also in part by Research Grant C-1780 from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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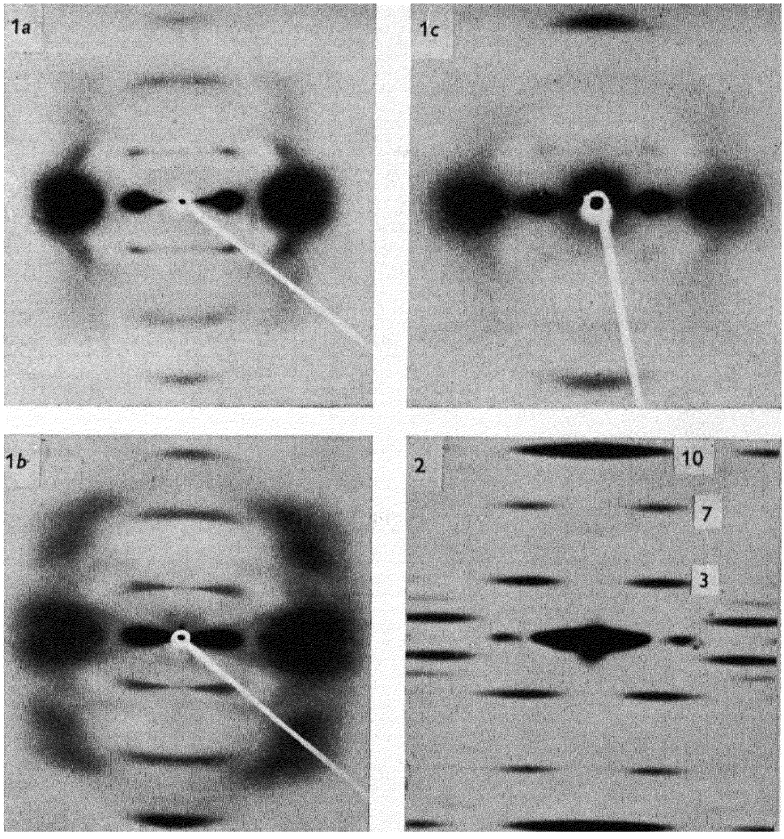
Note added to proof. Boedtker & Doty (*J. Amer. Chem. Soc.* **77**, 248 (1955)) have very recently confirmed the indications that acid dispersions of ichthyocol contain particles of mass per unit length around 100. More significantly, they find particles present which are shorter, more rigid, and more uniform in size than has been suspected. The new particle dimensions are: width 14 Å., length 2900 Å., and weight 30,000, corresponding approximately to expectations for tropocollagen. These specifications suggest that the tropocollagen particle may be an aggregate of three to four of the smaller units of weight *c.* 70,000, derived from studies on parent gelatin. As developed in this paper, the pertinent data now available more readily permit the smaller units to be combined in *series*, but future refinements of the crucial figures may allow the smaller units to be regarded as nearly fully extended polypeptide chains twisted in *parallel*. The series and parallel connexions offer the two most readily apparent extreme types of relationship between the two kinds of particulate unit.

EXPLANATION OF PLATE 1

Fig. 1. Diffraction patterns of kangaroo-tail tendon, illustrating (*a*) the effect of tension on the wide-angle diagram of dry material; (*b*) the influence of moisture and tension on the wide-angle diagram; (*c*) the result obtained from dry tendon under tension by use of Mo$K\alpha$ instead of the usual Cu$K\alpha$ radiation. Pattern *c* has been magnified from the original size to become comparable to *a* and *b*, which were originally photographed at a 5 cm. specimen-to-film distance. Fibre axes are vertical in all cases.

Fig. 2. The optical-diffraction pattern (transform) obtained for the Crick model for collagen, including effects of maximum (excessive) amounts of proline and of β-carbon atoms at all other side-chain locations. The transform is magnified to be comparable in size to fig. 1*b*. Layer-line indices indicate the interesting levels for comparison of near-meridional diffraction.



For explanation see p. 114

X-RAY DIFFRACTION STUDIES OF COLLAGEN FIBRES

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The high-angle X-ray diffraction pattern given by collagen fibres has been studied extensively for many years and indicates that the polypeptide chain configuration of collagen is distinct from those structures found in other fibrous proteins (Astbury, 1938). Collagen fibres usually display also a characteristic low-angle pattern. The chemical and physical significance of the X-ray data have been reviewed recently by Bear (1952). Since this review was published we have shown that the high-angle pattern could be improved if the collagen fibre were held under tension while in the X-ray camera and have described the main features of this pattern (Cowan, North & Randall, 1953).

The subject-matter of the present paper is divided into two main sections corresponding to different levels of organization within the collagen fibril. These are (i) large-scale or macroperiod structure, and (ii) the detailed structure of the highly ordered parts of the macroperiod. We shall describe some recent X-ray studies appertaining to each of these levels.

I. STRUCTURE OF THE MACROPERIOD

Observations with the electron microscope show collagen fibrils normally to consist of alternating regions of greater and less density, termed respectively bands and interbands. The predominant period of this banding is about 640 Å. Lower periods (200 Å.) are observed in developing collagen (Randall, Fraser, Jackson, Martin & North, 1952), and higher in precipitates from collagen solutions (Highberger, Gross & Schmitt, 1951). Sub-bands of axial length about 70 Å. are often visible within the main period.

Low-angle X-ray diffraction also has demonstrated the existence of a period of about 640 Å. From analysis of the low-angle diagram, Bear, Bolduan & Salo (1951) have concluded that along the length of the fibril exist alternate regions of less and greater order which they have associated respectively with the bands and interbands seen in the electron microscope; the high-angle diagram is thought to arise from the ordered interbands

only and is characteristic of a set of parallel units, protofibrils, about 12 Å. apart. They have also found that the macroperiod increases on wetting the fibre, though the high-angle meridional spacings do not change significantly; they therefore attributed the change in low-angle pattern to the ordering and elongation of the bands. Rougvie & Bear (1953) have shown the effect of moisture sorption on the X-ray reflexions to be consistent with such a two-'phase' system. It seemed useful, therefore, to compare the relative effects of fibre extension on the low-angle pattern, representing the whole structure, and the high-angle pattern, representing the ordered part of the structure, in order to determine whether this behaviour was compatible with a fibre having alternate regions of greater and less order.

The remainder of this section is a description of new data pointing to the existence of structural units shorter than the macroperiod and of lateral order greater than the individual protofibril.

The material used throughout this work was rat-tail tendon,* air-dried under slight tension before X-ray investigation; fibres of 100–150μ in diameter were employed, and were fastened in an adjustable metal clamp designed to fit inside the X-ray cameras. Normally, high-angle pictures were obtained by the use of a Unicam 6 cm. diameter cylindrical camera with 250μ collimator; higher resolution was attained with a flat plate camera having a 100μ collimator and 5 cm. specimen-film distance. Low-angle diagrams were obtained with a slit camera having an f.d. value of 2000 Å. (Bolduan & Bear, 1949; Norris, 1953).

Variation of X-ray spacings with fibre extension

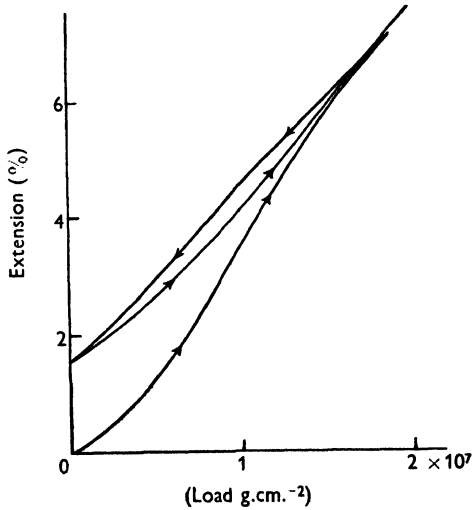
It is of interest to consider first the behaviour of a collagen fibre under load. The extension is found to consist of a true Hooke-law elasticity, together with a slow creep; the original fibre length is completely recoverable after a considerable period of time (perhaps several weeks). Text-fig. 1 is a stress-strain curve for a fibre which was extended, relaxed and immediately re-extended, and shows the relative effects of creep and elastic stretch.

The X-ray data were obtained on three specimens which underwent cycles of extension and relaxation; high- and low-angle photographs were both taken at various stages of the cycles. Text-fig. 3, in which the three symbols relate to the different specimens, shows that the low-angle spacing is always directly proportional to the length of the specimen and that the increase in spacing is about 90% of the specimen extension. That this factor is not due to slip is shown by the fact that points for both extension and relaxation fall on the same line; it is probably caused by straightening of the individual fibrils within the fibre.

* Afterwards abbreviated to R.T.T.

The spacing of the high-angle meridional arc varies in a more complex manner. When tension is completely removed from a stretched fibre it does not return immediately to its original length because of creep. Nevertheless, it gives a high-angle X-ray diagram characteristic of an unstretched specimen, as does a fibre which has been left without tension until it has returned to its original length.

Specimens taken through several repeated cycles of extension and relaxation, each cycle taking about a fortnight to complete, produce a given high-angle spacing at a greater fibre extension in each successive cycle.

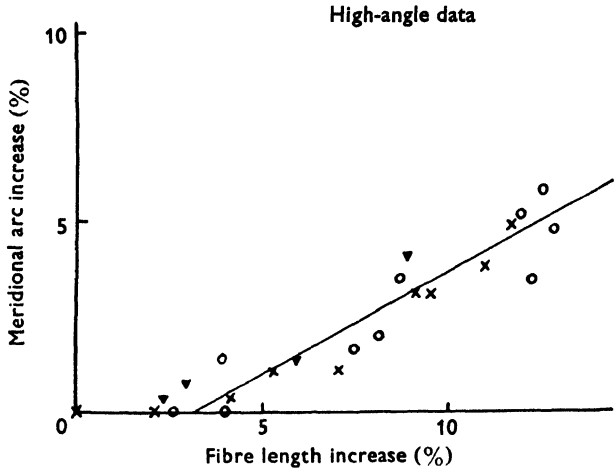


Text-fig. 1. Stress/strain curve for dry R.T.T. fibre.

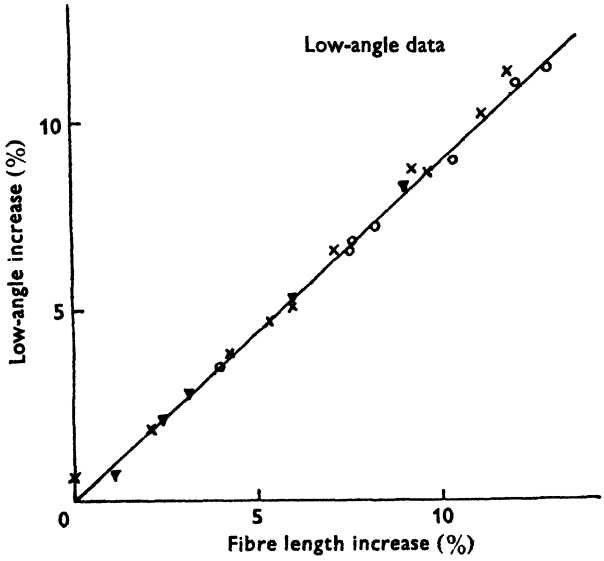
Thus the high-angle spacing appears to be linked with the elastic component of fibre extension rather than with the creep. Text-fig. 2 illustrates the variation of high-angle spacing with fibre length; the straight line drawn through points obtained during both extension and relaxation is a measure of the variation of spacing with the elastic component of extension. It is immediately obvious that the change in high-angle spacings is proportionately much less than the corresponding change in low-angle period; stretching the fibre sufficiently to increase the low-angle spacing by 10% causes a gain of only 4·3% in high-angle spacing. Part of this difference must arise from the increase in length resulting from the improved orientation which is obvious in the X-ray diagram. The observed creep might be due to the orientation process. The question arises as to whether the difference can be explained solely by orientation or whether it should be attributed to the existence of poorly ordered regions in the collagen fibre,

which may be extended more readily than the ordered region which produces the high-angle diagram.

If we suppose that orientation is the sole explanation, we find that it is necessary for the chains in an unstretched specimen to have a mean disorientation of at least 18° on the assumption that orientation is perfect in a specimen stretched 10%.



Text-fig. 2. Variation of 2·86 Å. arc with fibre extension.



Text-fig. 3. Variation of 640 Å. period with fibre extension.

It is possible to estimate the disorientation in a specimen from the angular spread of reflexions on the X-ray diagram; we have calculated the angular intensity distribution of the 10 Å. equatorial reflexion for both an unstretched and a 10% stretched specimen. Again assuming perfect orientation for the stretched specimen we are led to a maximum estimate for the mean disorientation of about 9°. Experimental errors tend to make this value an over-estimate.

The value is thus considerably less than that required by the stretching data, though the margin is not really sufficient to be absolutely certain that the two figures could not be reconciled. We have concluded therefore that it is highly improbable that orientation is the sole explanation of the effect and that the present results show the existence of two types of region, differing in their degree of order, to be very likely.

The size of the collagen unit

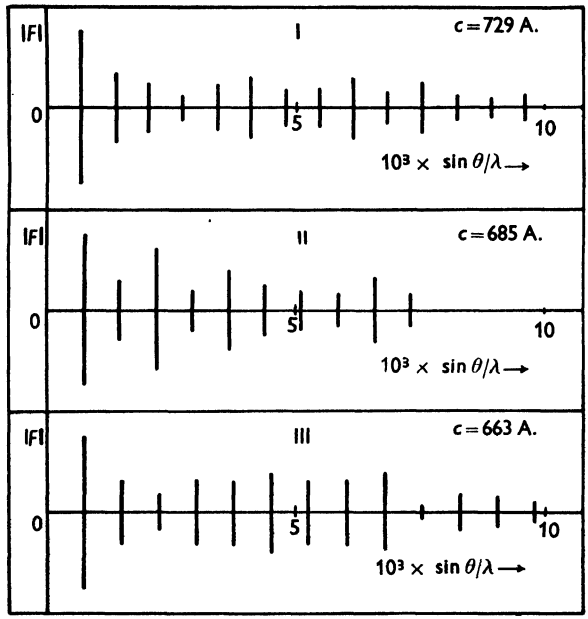
While the 640 Å. unit of collagen is the predominant feature of electron microscopic and low-angle X-ray investigations, there is considerable evidence from these fields of the occurrence of both larger and smaller structural units. We have therefore examined X-ray diagrams for evidence of the size of the diffracting unit.

Examination of the variations in intensity of the orders of the 640 Å. period gives some evidence of the presence of subunits. In Text-fig. 4 are plotted the observed F-values as a function of $\sin\theta/\lambda$, for air-dried, wet and P.T.A. stained fibres. If we attempt to trace the form factor for the air-dried fibres we find that the zeros fit in remarkably well with those expected for a spherical or ellipsoidal diffractor of length 210–240 Å. in the fibre direction. The rapid alternation of lower orders in the wet fibres indicates a larger unit, but of course the water and the heavy P.T.A. molecule in the stained fibres tend to mask the effect of the collagen alone. All three patterns, however, exhibit strong ninth and weak tenth orders which might be due to still smaller subunits about 70–80 Å. long.

In Pl. 2, fig. 3, is shown an X-ray photograph of vacuum-dried collagen taken in the camera of high resolving power with nickel-filtered Cu$K\alpha$ radiation. Round the centre of the film is a roughly ellipsoidal halo. From this low-angle scattering we have computed the size of the collagen 'crystallite', assuming it to be an ellipsoid of revolution, by the method of Guinier (1952). The dimensions thus found are about 200 Å. in the fibre direction and from 40 to 90 Å., according to the dryness of the specimen, at right angles. This measured width corresponds to between 3 and 7 times the probable chain diameter; it is possible that more drastic drying would result in splitting the structure into yet thinner units.

Recent measurements on the variations of electron density along the fibre-axis period, obtained from electron micrographs, are reported elsewhere in this volume by Randall, Kelly, Fitton Jackson, Burge & Booth, and are in reasonable agreement with our X-ray measurements.

It is noteworthy that Finean & Engström (1953) have obtained dimensions almost identical with ours, also by the low-angle scattering method, for the apatite crystals with which collagen is very closely associated in the structure of bone. The long axis of the apatite is parallel to the collagen fibre.



Text-fig. 4. Observed structure factors from low-angle diagrams.

Large-scale lateral order in native collagen

We have recently examined the X-ray diffraction patterns given by specimens of tendon which were removed from the rat tail and sealed into thin-walled glass capillaries before they had time to dry. Such specimens are much more characteristic of the natural state of collagen than fibres which have been so dried that the fluid which normally surrounds the polypeptide chains has been lost.

The remarkable feature of the resultant fibre diagram (Pl. 2, fig. 4) is the presence of a number of equatorial reflexions of large spacing. The usual inter-chain reflexion has split up into two components, one on the equator at 12·6 Å. and one just off it at 13·5 Å.

It is probable that some of the other spots may eventually be resolved into off-axis components. The equatorial or row-line spacings for the spots are given below, together with an indication of their intensities:

12·6	13·5	14·9	17·0	18·8	20·5	24·5	31	37	49 Å.
VS	VS	M	M	M	W	M	W	S	S

Reflexions of these spacings cannot be obtained by rewetting dried fibres, which is probably why they have not hitherto been observed generally. Corey & Wyckoff (1936) reported spacings within this range; however, the low value (10·9 Å.) for the interchain spacing indicates that their material was quite dry, and the higher spacings have been considered by other workers to arise from impurities.

The height of the spots on the 13·5 Å. row line above the equator corresponds to a spacing in the fibre axis direction of about 200 Å.; the recurrence of this figure is interesting, but its significance is not apparent at present. It may be found to represent the pitch of a super-helical configuration of the chains of the ordered region.

The native R.T.T. fibres give a meridional low-angle pattern similar to re-moistened dry fibres.

It is evident that the moisture present naturally in collagen contributes to a much higher degree of lateral order than had been supposed. As is common in the more truly crystalline proteins much of the order is lost on drying, a process which appears to be an irreversible one. We have unfortunately not yet had time to study the effects of swelling agents or of controlled drying on native R.T.T. An investigation of the change in X-ray diagram as water is gradually lost, i.e. the experiment of Rougvie & Bear in reverse, should prove interesting.

We have shown in this section that there is considerable evidence for the existence of different degrees of order within the collagen macroperiod. The diffuseness of the high-angle pattern itself points to the small size of the effective crystallite. The macroperiod, on the other hand, is so regular in its repeat that up to fifty orders of its fundamental spacing are clearly defined, so that there must in fact be considerable longitudinal regularity in even the less ordered parts of the fibril.

The X-ray data point to a diffracting unit about 200 Å. long, possibly consisting of three subunits; the evidence of electron microscopy also involves units of these sizes. The overwhelming intensity of the first order of diffraction of the 640 Å. period and the asymmetry of the banding of this period show that this larger unit is the true repeating entity of the fibre in its mature native state; it remains to be found, therefore, not only how the

subunits are built up from the molecular chains but also how the subunits themselves are arranged to form the macroperiod.

We shall now consider the structure of the well-ordered regions in greater detail.

II. STRUCTURE OF THE PROTOFIBRIL

A photograph of dry unstretched R.T.T. is shown in Pl. 1, fig. 1, for comparison with that of a specimen stretched 8% (Pl. 1, fig. 2). Text-fig. 5 is a table of the observed spacings of Pl. 1, fig. 2. The general effect of stretching is seen to be an improvement in orientation, resulting in reduced arcing of the reflexions. In particular:

(*a*) The 'meridional arc' at about 4 Å. is now seen properly to consist of two off-meridional spots.

(*b*) The only true equatorial reflexions are that at 10–11 Å. and its second order; the diffuse halo at about 4 Å. has become split up with maximum intensity off the equator.

The absence of meridional reflexions between the equator and the 2·95 Å. arc (the meridional arc sometimes seen on the 10 Å. layer line may well belong properly to the low-angle pattern) and the relationship

$$\frac{1}{2{\cdot}95} - \frac{1}{4{\cdot}27} \approx \frac{1}{10{\cdot}14}$$

between the spacings of the strong layer lines would be expected from a helical configuration with a unit repeating at heights of 2·95 Å. and a pitch of either 10·14 or 4·27 Å. The repeating unit may consist of one or more residues. The 10 Å. pitch could be produced by:

(i) A single chain having a lead (turn height) of 10 Å. and about $3\frac{1}{3}$ units per turn.

(ii) Two intertwined helices each having a lead of 20 Å. and about $6\frac{2}{3}$ units per turn.

(iii) Three intertwined chains each with a lead of 30 Å. and 10 units per turn.

(i) and (iii) each have an approximate pseudo-period of 30 Å. and (ii) one of 60 Å. A 4-Å. pitch might similarly be produced by one, two or three interwoven helices with leads of 4, 8 and 12 Å. respectively.

The table of observed spacings has been indexed according to layer lines of a 30 Å. period. Cohen & Bear (1953), who propose a helix of 10 Å. pitch, formally similar to (i), have indexed the reflexions of unstretched kangaroo tail tendon according to a 20 Å. period. For neither period is the fit exact, and in practice a helical structure may have a very long pseudo-period for which these systems of small layer-line indices would be approximations.

Though the extent of lateral order varies from native to dry material, there seems little doubt that in any case the equatorial reflexion at 10–15 Å. represents the spacing between adjacent planes of protofibrils. The individual protofibrils have no simple axial symmetry and would be expected to be arranged in hexagonal packing; they are probably packed this way in small bundles, the bundles themselves being ordered in native collagen and independent in dried material.

Layer line		Spacings (Å)
No.	Spacing (Å)	
0	—	11·4 5·7
3	10·14	7·27 4·58
4	7·18	5·47
5	6·07	3·80
6	5·05	4·68 3·63
7	4·27	4·18 3·46 2·83
—	2·95	2·95 (meridional arc)
10	2·95	2·65
12	2·58	2·35
13	2·30	} diffuse arcs (off meridian)
14	2·11	
16	1·91	
—	1·47	1·47 (second order meridional arc)

Text-fig. 5. Observed spacings from the X-ray diagram of dry R.T.T., stretched 8% (see Pl. 1, fig. 2).

The spacing 10·4 Å., which is the usually accepted value for dry collagen (we have observed a value of about 10·15 Å. for the intensity maximum in vacuum-dried R.T.T.), corresponds to an effective protofibrillar diameter of 12·0 Å. The two normal equatorial reflexions correspond to the $10\bar{1}0$ and $20\bar{2}0$ of the hexagonal system. A $11\bar{2}0$ reflexion would be expected from this system, but is not observed; its absence might be due to a minimum in the form factor of the individual protofibril occurring near this spacing. The swelling of collagen due to moisture results in a greater change in the spacing between chains than in the dimensions of the chains themselves, so that the reflexions move along the form factor. We have found from measurements of photographs having a range of equatorial spacings that the higher the spacing the greater is the ratio of the $10\bar{1}0$ to $20\bar{2}0$ intensity; this is consistent with the existence of a form factor minimum between these reflexions, so that, on swelling, the $10\bar{1}0$ spacing moves away from the minimum and the $20\bar{2}0$ towards it. The form factor for the distribution of intensity along the equator from a helical array of diffractors is a zero-order Bessel function; examination of the photographs suggests that the intensity minimum occurs at about 7·2 Å.; if this value corresponds to the first zero of the Bessel function, and if we may assume the diffraction pattern to

originate largely from main chain atoms concentrated near a certain mean radius, this radius must be about 2·7 Å., a value which is quite consistent with the packing of the protofibrils.

Assuming that the density of the crystalline region of the fibre does not differ greatly from the average for the whole fibre, the calculations of Pauling & Corey (1951) show that the 3 Å. repeating unit of the protofibril should contain three residues; if, however, there are large spaces between the bundles of protofibrils in dry material (for which the figures of density have been obtained) or the crystalline region has either a greater density than the average or contains mainly residues of less than average weight, four residues may occur in the repeating unit.

Structural models

At a previous meeting (Cowan *et al.* 1953) we discussed the several models which had been proposed for collagen to that date. Since then three more have appeared in the literature, due to Crick (1954), Ramachandran & Kartha (1954) and Huggins (1954). Each one of these structures has a number of undesirable features, that of Ramachandran failing to account for some of the main features of the X-ray diagram.

In the course of the last year we have built nearly one hundred models in attempting to find the structure of the protofibril, none of which have we considered satisfactory for publication. It may be that either the premises on which we have based the models or our methods of testing them have been wrong. In describing the work here we hope that other workers in the field will offer suggestions and criticism which may lead ultimately to a satisfactory solution.

We have approached the problem from two different angles: (i) to attempt to build up a structure on a preconceived architecture based on the observed X-ray spacings; (ii) assuming that the distinctive feature of collagen is its high proportion of proline, to examine the effect of introducing proline or hydroxyproline residues at intervals along known chains such as the α-helix.

(i) *The direct approach*

The principal dimensions to which we have attempted to build models have been discussed in a previous section. We have concentrated largely on helices of pitch 10 Å. consisting of one, two or three chains.

The dimensions of the *trans* amide residue were taken from Corey & Donohue (1950) and of the *cis* from Pauling, Corey & Branson (1951), only small deviations from these figures being allowed. Although proline dipeptides readily form diketopiperazines in which the residues must have

a *cis* configuration (Neuman & Smith, 1951), we have had a prejudice against the *cis* form as it has not been observed to occur elsewhere. The infra-red spectrum of collagen (Badger & Pullin, 1954) shows that the proportion of *cis* groups cannot be large if they are present at all. (The structures proposed by Crick and by Huggins both contain predominantly *cis* residues.) We originally made the following additional assumptions:

(1) That all residues should as far as possible be equivalent in preference to assuming any repeating sequence such as gly-pro-*R* or mixed *cis* and *trans*.

(2) That staggered configurations should be taken up by α-carbon atoms if possible.

(3) That a large proportion of the amide groups should be hydrogen bonded.

(4) That there should be 3 ± 1 amino-acid residues in the repeating unit.

We were unable to build structures of the desired dimensions in which all these assumptions held simultaneously. For example, a single chain structure approximately obeying (1) and (2) could be made readily, but it did not conform to (3) and (4). Subsequently (1) was dropped, and a number of mixed *cis* and *trans* structures were built, including a single-chain helix of pitch 10 Å. with two *trans* and one *cis* residue in 2·86 Å., but which had insufficient hydrogen bonding to prevent it from extending readily.

(ii) *The indirect approach*

The replacement by proline or hydroxyproline of other residues in an existing chain causes distortions because of their failure to make N—H···O bonds, their bulk and the configuration which they force upon the bond angles at the α-carbon atom. Placed regularly in a helix, the distortions cause the development of a super-helix. We investigated such structures systematically, but found no suitable model to produce the required meridional arc and layer lines.

In testing model structures, use was made of a Lipson optical diffraction apparatus.

It is possible that we are mistaken in interpreting the strong layer-line spacings as the pitch height, since it might be an accidental reinforcement arising from a particular arrangement of side-chains. Concentration on the main chain configuration rather than the effect of side-chains may also have proved misleading. Finally, the assumption that a single structure can be built to account for the entire high-angle diffraction may well not be justified.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. X-ray diagram of dry unstretched R.T.T.

Fig. 2. X-ray diagram of dry R.T.T., stretched 8%.

PLATE 2

Fig. 3. High resolution X-ray diagram of dry R.T.T., stretched 11%, displaying low-angle scattering.

Fig. 4. High resolution X-ray diagram of undried R.T.T., displaying equatorial reflexions of large spacing.

PLATE I

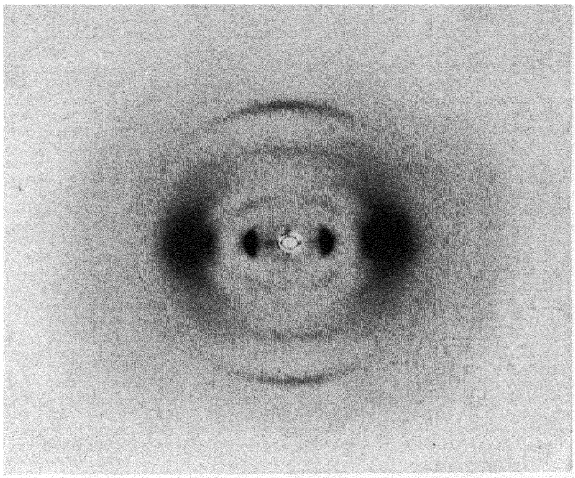

Fig. 1.

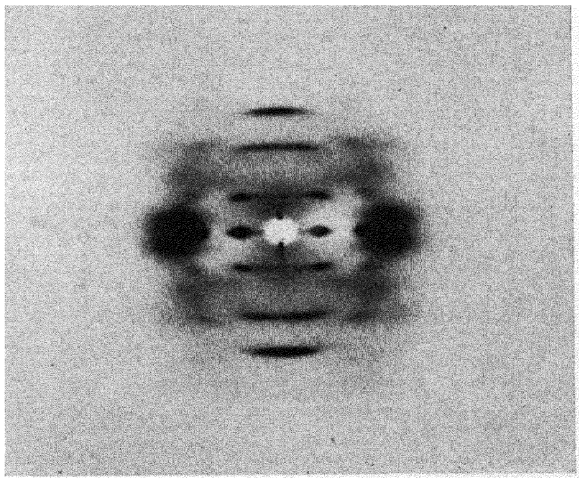

Fig. 2.

For explanation see p. 126

PLATE 2

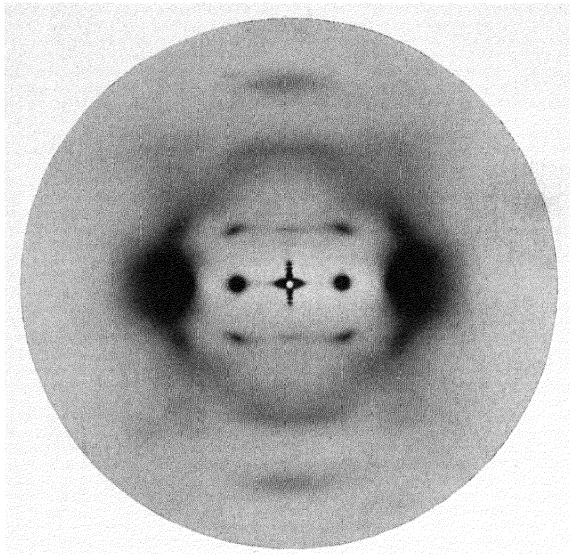

Fig. 3.

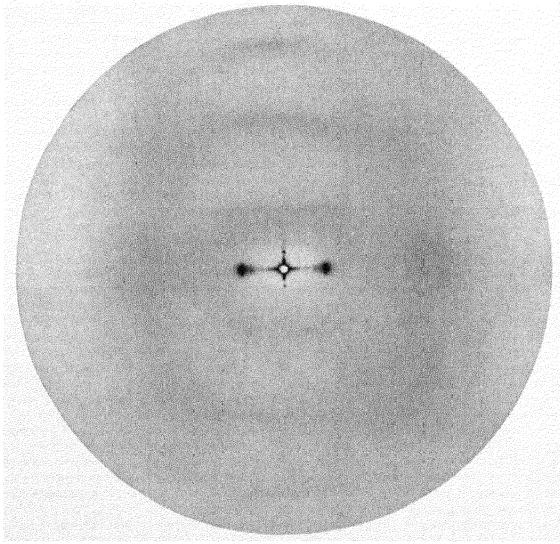

Fig. 4.

For explanation see p. 126

OBSERVATIONS ON NATIVE AND PRECIPITATED COLLAGEN

By J. T. RANDALL, F. BOOTH, R. E. BURGE, S. FITTON JACKSON and F. C. KELLY

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INTRODUCTION

The collagen system is a complex unit of fibrogenic cells, extracellular fibrils and ground substance interspersed to some extent with intercellular fluid and blood vessels. In another paper in this symposium new experimental evidence is being presented on fibrogenic cells, their cytoplasmic contents and biochemical constitution; in the present paper we are largely concerned with the more physical aspects of fibres, fibrogenesis and structure. It would indeed be rash to say that most of the striking facts about the fine structure of collagen have now been found, since new facts are still forthcoming at a considerable rate. But it is true that many important broad features are now known, at least as regards fine structure seen in the electron microscope. The position with regard to X-ray diffraction is more uncertain, since it is here—as much as in any other aspect of the subject—that new facts are continually coming to light, especially in the high-angle pictures.

Studies of collagen fibrils in the electron microscope have for the most part been aided by the use of metal shadowing and electron staining (e.g. phosphotungstic acid) techniques. In consequence, much of the information so far published is useful only in so far as it gives information about the periodicity along the fibril and the fibrillar contour, which may be smooth or corrugated in various ways. The use of these important ancillary methods obscures the true density of scattering material along the length of the fibrils. And yet, if electron microscopy is ever to be linked with the important results of low-angle X-ray diffraction (cf. Bear, 1952), it is above all the electron density of the native fibril that is required. (The processes inherent in the preparation and examination of specimens in the electron microscope may defeat this object, but at least the attempt should be made.) The first part of this paper, therefore, relates largely to the study of unstained, unshadowed, native collagen fibrils and attempts to correlate these results with the X-ray low-angle determinations.

In the second part of the paper we turn to problems of the precipitation of collagen from acid solution. Although this has been known for many years, the chief emphasis of recent work in this field has lain in the demonstration, again by the use of the electron microscope, that *fibrils*, exhibiting substantially the same degree of fine structure as native fibrils, may be produced by the action of simple ionic salts and pH changes on collagen solutions. The use of much more complex molecules (e.g. glycoproteins, chondroitin sulphate) has given more complex precipitates in which fibrils showing much longer axial periodicity are common. A careful study of the literature has made it clear that the precise conditions under which these various complex phenomena are found have never been published; and it is doubtful, owing to the complexity of the problem, if they are known. In this section of our paper we therefore try to examine what are the important factors in the fibrogenesis of collagen from solution both experimentally and theoretically. This analysis is preliminary and can only at this stage be said to be a guide to the general principles involved.

In the third section of the paper we are concerned with the structural and biochemical features of complex fibril formation by some of the agents already referred to. This work is an attempt to define the conditions of formation of long-spacing fibrils and segmented fibrils more closely. In the notation of Schmitt and his collaborators (1951, 1953) these particular fibrils are referred to as FLS and SLS respectively; this notation is followed in this paper.

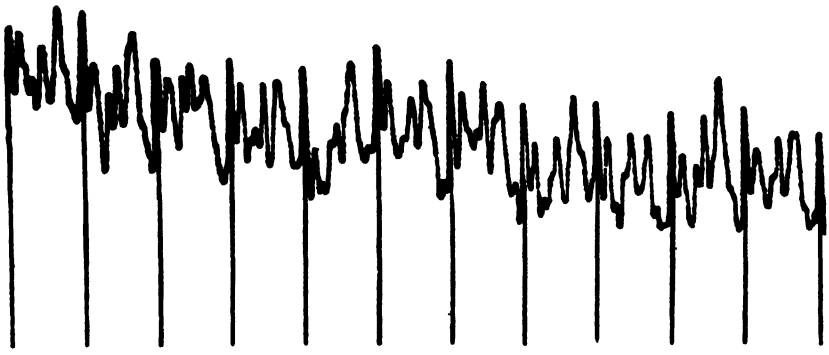
PART I

Studies of native collagen fibrils in the electron microscope

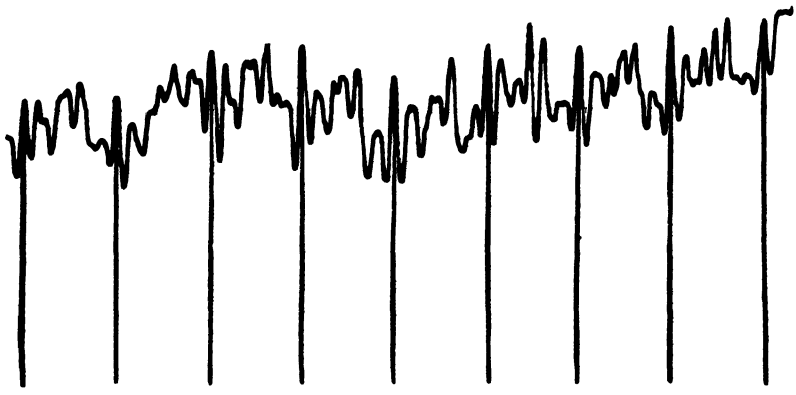
The results presented here depend largely on the fact that under specific conditions the blackening of a photographic plate by an electron beam is directly proportional to the logarithm of the time of exposure of the plate to the beam. The constructed H–D curve for the plates we have used is linear between photographic densities 0·9 and 3·7, and the densities of all the plates examined were in this range. It was also assumed that the scattering cross-sections of carbon, nitrogen, oxygen and hydrogen atoms (which are the predominant constituents of collagen) were the same under the examining conditions of an electron beam sharply limited by an objective aperture (Coupland, 1954). Providing these assumptions are valid the photographic density differences between points on a collagen fibril and the background of the electron micrograph are directly proportional to the concentration of scattering material at those points in the fibril.

Longitudinal density fluctuations. Our measurements of the usual band structure of unstained, unshadowed native collagen from fowl-neck tendon

(Text-fig. 1), and from rat-tail tendon measured along the fibril axis, demonstrate that the similarities between these two materials are very close and the differences small. Text-fig. 1 shows density traces obtained by scanning the fibrils of fowl-neck tendon (F.N.T.) and rat-tail tendon (R.T.T.) longitudinally. The *d*-band (Schmitt and Gross notation, 1948) in both



(*a*)



(*b*)

Text-fig. 1. Densitometer traces of (*a*) fowl-neck tendon, (*b*) rat-tail tendon, obtained by longitudinal scanning of unstained, unshadowed material. The *d*-bands of the main period are marked by vertical lines; up to 8 bands are visible in each period.

traces is strongly marked and supplies the natural position from which to measure the main period. The traces show some small differences:

(i) The a_2 band (Grassmann notation, 1953) is absent in R.T.T., while the a_1 band is very well differentiated and of width approximately 80 Å. In F.N.T. both a_1 and a_2 are present and the total *a* band is very much more prominent and of width ~ 200 Å; the *a* band may spread into the region of the e_2 band which has not been resolved in this material.

(ii) The b_2 band in R.T.T. is more prominent than that in F.N.T. and considerably wider—approximately 75 Å. compared with 45 Å., though their relative positions in the 640 Å. period are the same.

(iii) The density minimum separating the b_2 band from the c_1 band in F.N.T. is very much more pronounced than in R.T.T.

In this discussion we have referred to fine-structure *bands* within the main period; these *bands* alternate with less dense regions. The complete series of alternating regions of greater and lesser density may be referred to conveniently as *striations*. The bands appear on the density trace superimposed on the central cylinder of the fibril as a fluctuation of ± 10% of the mean fibril density. The principal band widths for these materials in Å. (normalized to a period of 640 Å.) are shown in Table 1, while in Table 2 are given details of the percentage separations of density maxima for 8-banded specimens of R.T.T. collagen. Measurements of fibrils with a smaller degree of fine structure fit in well with the figures of Table 2, while comparison of our results with those of Schmitt & Gross (1948) in Table 3 shows a very close agreement between the two investigations.

Table 1. *Comparison of band widths in native collagens*

	a	b_1	b_2	*c*	*d*
Rat-tail tendon	70	79	71	74	98
Fowl-neck tendon	200	78	47	90	78

Table 2. *Band structure in native rat-tail tendon*

	d	e_1	e_2	*a*	b_1	b_2	c_1	c_2	*d*
	0	11·2	18·4	38·9	52·3	64·0	74·6	82·5	100%
	0	11·2	20·2	39·5	52·5	65·2	—	82·1	100%
	0	13·4	20·5	39·5	53·5	63·7	78·5	86·2	100%
	0	13·1	21·1	40·0	55·0	65·7	78·0	83·0	100%
	0	—	18·6	40·4	56·7	65·2	79·2	84·8	100%
	0	14·5	20·6	39·6	53·4	62·9	74·3	80·6	100%
	0	13·7	21·5	41·8	54·2	64·3	79·4	83·4	100%
Average	0	13·0	20·2	40·0	54·1	64·3	76·9	83·4	100%
Standard deviation		1·2	1·0	0·8	1·5	0·9	2·2	1·7	
Equivalent in Å.		83	129	256	346	412	492	534	640

Table 3. *Reproducibility of band structure in native rat-tail tendon*

Source	*d*	e_1	e_2	*a*	b_1	b_2	c_1	c_2	*d*
Schmitt and Gross R.T.T. (P.T.A. stain)	0	—	18	37	—	60	—	82	100%
8-banded R.T.T.	0	13·0	20·2	40·0	54·1	64·3	76·9	83·4	100%
5-banded R.T.T.	0	—	19·0	39·0	54·2	—	—	83·5	100%
5-banded R.T.T.	0	—	20·7	43·5	—	62·9	—	83·2	100%

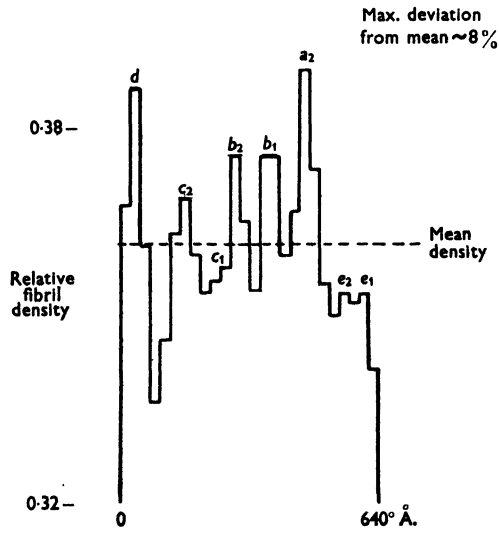
Table 4 gives a comparison of our measurements for R.T.T. and F.N.T. with those of Grassman (1953) on cat-tail tendon, and there is again remarkably good agreement.

Table 4. *Band structure in collagen from different sources*

	d	e_1	e_2	a_1	a_2	b_1	b_2	c_1	c_2	d
R.T.T.	0	13·0	20·2	—	40·0	54·1	64·3	76·9	83·4	100%
F.N.T.	0	16·4	—	29·8	37·7	54·4	65·1	74·9	86·3	100%
Cat T.T.	0	16·9	—	34·1	41·2	55·2	63·6	75·5	85·9	100%

The relationship between electron microscopy and low-angle X-ray diffraction of collagen

The results that have just been described have been used to calculate the longitudinal variation of fibril density of rat-tail tendon shown in Text-fig. 2, which may then be used to calculate the X-ray scattering pattern at low angles.



Text-fig. 2. Diagram of the relative variation of fibril density within the striations ofrat-tail tendon.

The integrated intensity I_k of the kth layer line of the calculated diffraction pattern is given by $I_k = \alpha |F_k|^2$,

where
$$F_k = \int_0^{640} N(z) \exp 2\pi i z d z, \qquad (1)$$

where $N(z)$ is the periodic variation in density along the fibril axis z which is obtained from the densitometer trace. For constant layer-line lengths α is a constant.

This work is also at an early stage, since to reproduce analytically the fluctuations in intensity of the layer lines in a low-angle X-ray pattern from a collagen fibre, the density function $N(z)$ must be known with considerable accuracy. The function shown in Text-fig. 2 is the average variation obtained from an examination of several fibres, but this does not mean necessarily that it represents the true variation of fibril density within a 640 Å. period. The results obtained indicate that agreement with the experimental intensities for all layer lines can only be achieved by considering the system as a whole and not merely the diffraction from single fibrils with a given structure. In addition, various matters yet require attention; for example, it is important to make such comparisons for fibrils which have undergone the same degree of stretch and desiccation. At the present time there is no good evidence that the conditions of observation in the electron microscope are strictly comparable with those of the X-ray investigations.

PART II

Fundamental problems in the precipitation of collagen fibrils

Preliminary considerations. The earliest known work on the study of acid solutions of collagen in relation to precipitation goes back at least as far as the beginning of the century (Zacchariades, 1900), and much interesting work was done on the nature of the precipitates, using the light microscope by Nageotte (1927) and Faure-Fremiet & Garrault (1937). From the point of view of fine structure these investigations had little meaning, and it was not always easy to distinguish between a gelatinous precipitate and a fibrous one. Wyckoff & Corey (1936) were able to show that the high-angle X-ray diffraction pattern of precipitated collagen was substantially the same as that of native material, while Schmitt, Hall & Jakus (1942) were the first to show that banded fibrils could be obtained in the electron microscope by this means. Apart from their intrinsic interest such experiments may eventually throw light on the physical mechanism of fibrogenesis in living organisms. Before this can be achieved, however, much work, both experimental and theoretical, remains to be done. A study of the literature shows that remarkably little has been published concerning the precise conditions under which precipitation may occur, the work has been largely qualitative in character (Noda & Wyckoff, 1951; Vanamee & Porter, 1951; Gross, 1952), and this is undoubtedly due to the complexity of the problem. A little thought shows us that the number of parameters involved in the simplest possible experiment is large and the following would seem to be important:

(*a*) Concentration of protein solution.

(*b*) Change of pH: initial and final values are of importance.

(*c*) Rate of change of pH.

(*d*) Ionic strength $\Gamma/2$ of the added salt (and possibly the rate of its removal).

(*e*) Valency of the ions of the added salt.

(*f*) Temperature.

This list ignores the difficulty of obtaining identical protein solutions on different occasions. The complexity of the problem is perhaps still more clearly brought out by considering two parameters: concentration of protein and pH in relation to the amount of precipitate. (This implies that we fix the ionic strength and valency of the added ions and carry out the experiment at one fixed temperature.) If experiments were carried out to study these three variables we should probably find that a ternary diagram would enclose areas of fibrous and non-fibrous precipitate; it is impossible at present to predict what particular values of these parameters will give good fibrous precipitates. On grounds to be discussed later, however, it seems likely that low concentrations of protein will favour fibre production rather than a structureless gel.

Experimental results

(i) *Methods of precipitation.* An extensive series of experiments is now being carried out in an attempt to determine precisely the conditions of precipitation so that the results may eventually be compared with theory. As the experiments are not yet complete the results will not be given in detail. Solutions of collagen in acetic and in sulphuric acids have been studied at various values of protein concentration in a number of different ways:

(1) Acid solutions of collagen have been dialysed against distilled water of pH 7.

(2) Acid solutions of collagen containing salts of known ionic strength have been dialysed against water of pH 7 ($\Gamma/2$ decreasing with time).

(3) Acid solutions of collagen have been dialysed against water of pH 7 containing neutral salts of known ionic strength ($\Gamma/2$ increasing with time).

(4) Acid solutions of collagen containing neutral salts of known ionic strength have been dialysed against water of pH 7 containing an identical salt at the same ionic strength ($\Gamma/2$ constant).

(5) Acid solutions of collagen to which basic salts have been added have been dialysed against water of pH 7.

The protein concentrations in these experiments have ranged from 0·1 to 0·002%; the ionic strengths of added salts from $\Gamma/2 = 0{\cdot}05$ to $\Gamma/2 = 0{\cdot}30$. Experiments have been carried out with mono-, di-, tri- and tetravalent ions.

More information concerning the charge on the molecule is being sought electrophoretically to determine how mobility depends on pH for different salt concentrations. In previous work already cited precipitation has largely been carried out under conditions similar to (4) and (5) above, and it is well known that fibrils with fine structure can be obtained using these methods (Pl. 2, fig. 1).

No results corresponding to conditions (1) have previously been reported. We have found that (i) a 0·002% solution of rat-tail collagen in 0·001 M-sulphuric acid when dialysed against conductivity water of pH 7 gives beautifully normal banded fibrils (Pl. 2, fig. 2) and (ii) that a 0·018% solution of swim-bladder (*Polynemus* sp.) collagen in acetic acid dialysed against water of pH 7 produces similar well-formed fibrils (Pl. 2, fig. 3).

It appears from these results alone that ionic salts are not required in order to produce collagen fibrils from solution. This result is of considerable significance in relation to the physical mechanism of fibril formation and is discussed later.

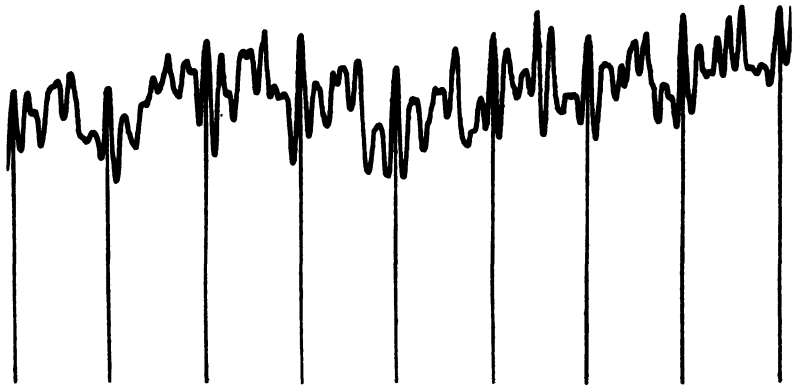
(ii) *Fine structure of precipitated fibrils.* It is not possible here to go into details, and one or two examples only can be given to show the general trend. In Table 5 are compared the fine structure observed for native rat-tail tendon and for 6- and 8-banded precipitated material. Although there are small differences in detail there appear to be good grounds for concluding that collagen fibrils precipitated by pH rise or with the aid of salts have the same fundamental structure as the native material, while the fine structure of the precipitated material appears to be sharper in character and each main period is more readily distinguished (Text-fig. 3).

The theory of coagulation

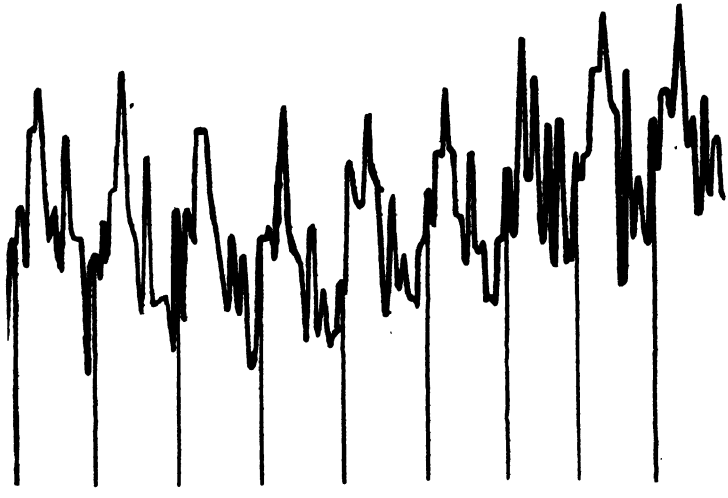
The form of the collagen molecule in solution. It will be useful before considering a more exact theoretical approach to the problem of precipitation to consider the form of the collagen molecule in solution. The evidence of light-scattering measurements (e.g. M'Ewen & Pratt, 1953) and other considerations make it reasonable to assume that collagen molecules in acid solution consist of randomly coiled extended chains with a net positive charge. At the isoelectric point (at, or probably below, which collagen precipitation takes place) there will be equal numbers of positively and negatively charged groups. The positively charged groups are attached to the residues of lysine, arginine and histidine; the negative carboxyl groups are those of glutamic and aspartic acids. For electrostatic reasons it also seems reasonable to postulate that these positive and negative charges will alternate along the length of the chain.

Table 5. *Comparison of fine structure of native and precipitated collagen*

Material	Method of precipitation	Fine structure in percentage of main period									
		d	e_1	e_2	a_1	a_2	b_1	b_2	c_1	c_2	*d*
Native rat-tail tendon		0	13·0	20·2	—	40·0	54·1	64·3	76·9	83·4	100%
6-banded precipitate	pH rise by water	0	15·0	—	—	38·9	54·0	71·3	—	85·0	100%
8-banded precipitate	Sodium acetate	0	11·9	21·4	33·8	46·2	56·9	65·2	—	85·5	100%



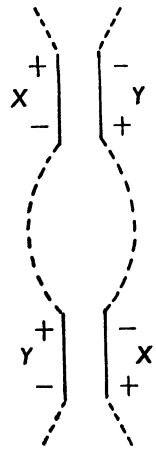
(*a*)



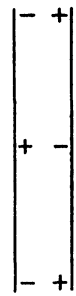
(*b*)

Text-fig. 3. Densitometer traces of (*a*) native rat-tail tendon and (*b*) a salt-precipitated fibre from a solution of rat-tail tendon which show that the two materials have the same fundamental structure.

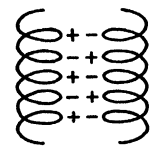
We also infer from experiment that fibrils are formed from the conjunction of the polypeptide chains; for theoretical purposes it might be adequate to consider the chains as straight (Text-fig. 5*a*). The proportions of positively and negatively charged residues in collagen (even assuming ionization of all the groups) is rather small, and amounts (by weight) to 9% for the basic and 11·6% for the acidic; the numbers of residues per 10^5 g. protein are 94·6 basic and 125·2 acidic. These figures are based on the work of Bowes & Kenten (1948). Electrostatically speaking one part of a single and flexible polypeptide chain of this constitution could interact



Text-fig. 4.



(*a*)



(*b*)

Text-fig. 5.

Text-fig. 4. Possible chain-to-chain interactions of appropriately charged portions of the chains.

Text-fig. 5. Possible interactions of positive and negative charges on adjacent (*a*) extended chains, (*b*) helical chains.

with another part, but only over a moderate fraction of the initial chain length. Chain-to-chain interactions would be similar provided the appropriately charged portions could approach each other sufficiently closely, possibly as shown in the diagram (Text-fig. 4). The interactions would not necessarily be so direct in practice because of the existence of adsorbed water molecules or of an ionic double layer which would drastically reduce any overall attractive force.

It does not, however, seem adequate to consider the interactions of comparatively extended simple chains. The X-ray evidence concerning the ultimate chain structure of collagen fibres is by no means certain. If, however, we accept the existing evidence that the structures of the native fibre and the precipitated fibre are identical, and we further agree that the

chains are helical, then positive and negative charges on any given chain may approach each other much more closely in a helical model (Text-fig. 5*b*).

It is necessary to ask how these conceptions fit in with current ideas on the structure of collagen. The nearest approximation we have been able to make to the structure (Cowan, North & Randall, 1953 and this volume) is a 3-chain helix having a pitch of 31 Å. with 10 residues per turn. Having regard to the chemical composition this means that there would be approximately one positive and one negative charge per turn of each chain of the triple helix. More accurately, there would be 8 charges of each sign (per chain) per 96 residues, i.e. 8 per 10 turns of 310 Å. axial length of helix. The total for the 3-chain helix is therefore 24 of each sign per 10 turns. These values have been estimated on the assumption of complete ionization of the acidic and basic groups near the isoelectric point. These broad considerations provide a model; we must now examine whether theory can bring out the essential features of coagulation of such long-charged molecules.

The theory of coagulation. The physical problem before us is to understand the solubility properties of collagen in relation to the concentration of protein, ionic strength of salt solution, temperature, etc. We also wish to know why, under some conditions, ordered precipitation of fibrils takes place rather than the formation of a gel. In the present state of our knowledge, theory cannot give more than preliminary answers to these questions.

The theory of the colloidal flocculation of inorganic lyophobic sols has already been fairly fully worked out for spherical particles and shows reasonable agreement with experiment. The early attempts in this field are due to Schmolukowski (1916, 1917), who was handicapped by lack of knowledge of the laws of force between interacting particles. He postulated that when the centres of interacting particles were less than a distance R apart there was infinite attraction. Nowadays it is possible to attack this problem more thoroughly. Although most calculations so far made have been for lyophobic colloids it may be expected that calculations for lyophilic systems will not differ in principle from those to be outlined here.

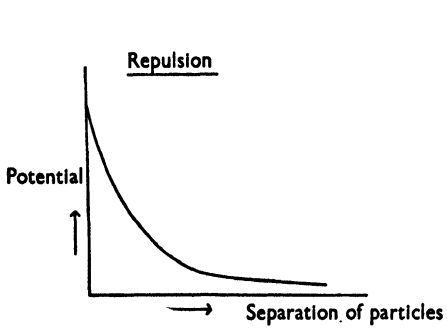
The initial interaction of the particles arises from two main effects:

(*a*) Van der Waals forces which are always attractive.

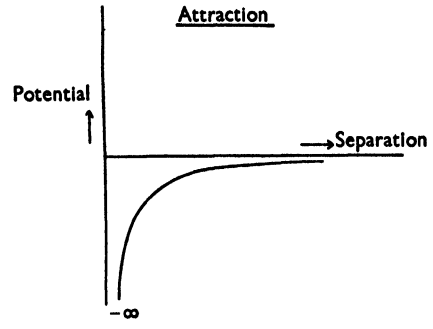
(*b*) Electrical interactions due to surface charges on the molecules which are modified by double-layer effects and adsorption on the molecular surface.

Such calculations do not at first sight seem to be very relevant to the collagen problem, but it turns out for the following reasons that these considerations are also of importance in the problem under investigation.

The repulsive and attractive forces for spherical particles are always of the form shown in Text-figs. 6 and 7. The repulsive force has a finite value at the origin, whereas the attractive forces are infinite. Furthermore, the repulsion falls away more rapidly than the attraction owing to double-layer effects. The result of this is that we always get curves of the *total* potential of the form shown in Text-fig. 8, which has three distinctive



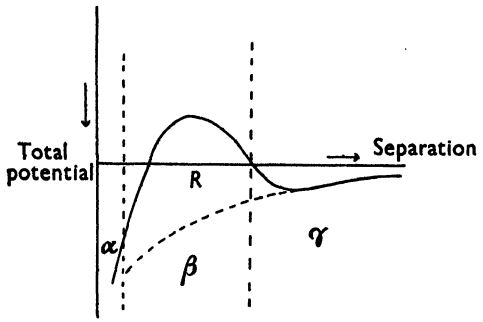
Text-fig. 6.



Text-fig. 7.

Text-fig. 6. The potential energy curve for repulsive (Coulomb) forces for spherical particles.

Text-fig. 7. The potential energy curve for attractive (van der Waals) forces for spherical particles.



Text-fig. 8. The combined potential energy curve for repulsive and attractive forces for spherical particles.

regions marked α, β and γ on the curve. In the region α the total potential is always attractive, while in region β it may be either repulsive or attractive. In region γ the total potential always results in weak attraction which gradually diminishes to zero for large distances. Except for small particles (< 10 Å. in radius) the rate of coagulation is very sensitive to the curve in region β. If the maximum lies below the abscissa coagulation occurs very rapidly. Hence it is quite a good approximation to say: If the V curve is always below the axis the sol will be unstable; precipitation or coagulation will set in.

If the V curve rises above the axis the sol will be fairly stable. Mathematically, the conditions are simply that for

$$\frac{dV}{dr}=O \quad \text{and} \quad \frac{d^2V}{dr^2} \text{ negative (so that the point is a maximum),}$$

$$V=O. \tag{2}$$

This condition greatly simplifies the whole problem and allows us to ignore collision dynamics and concentrate on the calculation of mutual interaction.

This suggests that for the collagen problem we must derive the potential between two long cylinders with the correct charge distributions for various relative orientations and separations. We may then use the criteria of (2) to decide on the conditions for precipitation or stability, noting that V will now be a function of orientation as well as of separation. There are various interesting possibilities:

(*a*) Attraction between the molecules in all relative positions. This would result in precipitation with no ordered structure at all.

(*b*) Attractive forces may exist only if the molecules are parallel to one another, in which precipitation would occur in the fibrous form. Van der Waals forces will always tend to orientate neighbouring molecules (region γ, Text-fig. 8).

(*c*) The forces may be repulsive in all positions in which case the solution will be stable.

It is also clear that an alternating or stepwise distribution of charge (as discussed in the last section) may be of vital importance in forcing molecules into parallel array; e.g. if the two helical molecules of Text-fig. 5(*b*) are parallel they will attract each other strongly. If the molecules are not parallel the total attraction is much reduced. An interesting feature of this model is that there will clearly be a turning moment tending to align the molecules, and this moment will increase very rapidly as the angle between the molecular axes decreases. It is perhaps useful here to give one example of the type of calculation that has been made. Before doing so it is necessary to stress the tentative nature of this approach. There are not at the present time enough experimental data to tell us whether this theory is correct; the pH experiments, however, emphasize the importance of charge and suggest that the general theoretical approach is sound.

For the purpose of illustration we shall mention the results of calculations on two long thin molecules (rods) at an angle ϕ to each other, each molecule being *uniformly* charged: both molecules of course have a charge of like sign. (The case of periodic alternating charge, while more interesting, is more difficult mathematically and has not yet been completely solved.)

If R is the minimum separation between the rods and the van der Waals law of attraction is λr^{-6} per atom, where ρ is the number of atoms in each rod per unit length, then the mutual van der Waals potential is given by

$$V_w = -\frac{3\pi\lambda\rho^2}{8R^5}, \tag{3}$$

Now consider the electrical energy of interaction arising from the supposedly smooth charge distribution postulated above. On the hypothesis that the charge (not the potential) on each molecule is fixed the electrical potential at a point P is given by

$$\psi(P) = 2Q(K_0(\kappa r_1) - K_0(\kappa r_2))/\epsilon, \tag{4}$$

where Q is the charge per unit length on each molecule and r_1 r_2 are the lengths of the perpendiculars from P on to the rods; $K_0(\kappa r)$ is the appropriate Bessel function and κ is the familiar parameter from the Debye–Hückel equation. The electrical interaction between the two rods is given by

$$V_e = 2Q\int_0^\infty dy(\psi_R(y) - \psi_\infty(y)), \tag{5}$$

where $\psi_R(y)$ is the potential at a point y on the inclined rod when the separation is R and y is measured from the position on the rods at minimum separation.

This leads to a value of V_e given by

$$V_e = \frac{2\pi Q^2}{\kappa\epsilon \sin\phi} e^{-\kappa R}, \tag{6}$$

and the total potential (from (3)) is

$$V = \frac{1}{\sin\phi}\left[\frac{2\pi Q^2}{\kappa\epsilon \sin\phi} e^{-\kappa R} - \frac{\pi\rho^2\lambda}{2R^4}\right] = \frac{W(R_1\kappa)}{\sin\phi}. \tag{7}$$

For the dependence of V on ϕ we have

$$\frac{\partial V}{\partial\phi} = -\cos\phi W(R_1\kappa)/\sin^2\phi. \tag{8}$$

This shows that if the overall potential is attractive then $\partial V/\partial\phi$ is positive and increases as ϕ decreases; in other words, there is a couple tending to align the molecules and the moment increases rapidly as ϕ decreases. On the other hand, if the overall potential is repulsive the effect is to throw the molecules out of alignment.

Summarizing we may say:

(i) Further analysis shows that for this particular model there seems to be no strong preference for fibrous precipitation rather than a random gel.

(ii) For the helical model attractive forces also tend to bring the molecules into alignment, and disorientate if the forces are repulsive.

(iii) If each helix carries an array of charges of both signs then these simple results do not necessarily apply, since the alternation of charges gives an additional short-range attraction between the helices.

The theoretical approach which has been made in this paper to the problem of collagen precipitation is not specific for collagen and could equally well apply to other long charged molecules in solution. It is hoped to test these ideas further, both on collagen and other polyelectrolyte systems. On this theory it is of interest that there is no obvious reason why a neutral salt should have any positive influence on fibril formation, since any double-layer would reduce the interaction between fibrils. The experiments recorded earlier show that pH change is the largest single factor in fibril formation from solution. This can only mean that the electric charge and its distribution on the molecule are the primary factors in the phenomenon and this is just what the preceding theoretical analysis has assumed.

PART III

Aspects of complex collagen fibril formation

In this section of the paper, however, we are concerned with some of the structural features, methods of formation of, and conditions of interconvertibility of, long-spacing fibrils (FLS) and segmented fibrils (SLS) which have been referred to in previous publications (Highberger, Gross & Schmitt 1951; Schmitt, Gross & Highberger, 1953; Randall *et al.* 1953).

Fundamentally it is necessary to distinguish between (i) fibril reconstitution by pH rise alone, (ii) fibril reconstitution by the use of salts, (iii) FLS complexes formed with other macromolecules such as glycoproteins, and (iv) SLS complexes formed either by manipulation of native material or the use of adenosine triphosphoric acid. (i) is the nearest approach to the theoretical considerations given earlier in this paper, while if fibrils are formed under condition (ii) (hitherto the usual method) the ions forming the ionic double layer are likely to remain attached to the molecules when fibrils are formed. Some theoretical ideas concerning the formation of complexes between collagen and poly-anions have already been outlined (Fitton Jackson & Randall, 1953) and are too involved for further discussion here. It seems probable that segmented long-spacing complexes may be formed under slightly less complicated conditions, e.g. by the use of a comparatively small anion such as ATP in the acid form.

Methods of formation and conditions of interconvertibility

The method by which we obtained SLS material from extracts of young rat skin (Randall *et al.* 1953) was essentially similar to that employed by Highberger *et al.* (1951) to obtain a preparation of FLS fibrils. However, the precipitate formed on dialysis of the phosphate extract against water was re-extracted with a citrate buffer of higher ionic strength ($\Gamma/2 = 0{\cdot}8$ instead of $\Gamma/2 = 0{\cdot}2$), and this citrate extract was then dialysed against slightly acidulated water of a pH between 4 and 5. We have found that

(*a*) If citrate buffer of high ionic strength ($\Gamma/2 = 0{\cdot}8$, pH 3·8) is used as an extractant and the extract is dialysed against acidulated water (pH 4–5) the precipitate which forms contains SLS and no FLS.

(*b*) On the other hand, if citrate buffer of lower ionic strength ($\Gamma/2 = 0{\cdot}2$, pH 3·8) is used as an extractant and the extract is dialysed against neutral water (pH 7), the precipitate which forms contains FLS and no SLS.

All experiments were carried out using skin from 6-week-old rats from the stock of the National Institute for Medical Research, Mill Hill. All preparations were made at 4° C. As a result of experiments covering a range of values of ionic strength of the citrate buffer and a range of values of the water pH, it seems that the value of the water pH against which the extract is dialysed is of greater importance than the strength of citrate solution in determining the form of the product.

Extension of the experiments summarized in (*a*) and (*b*) above show that the two forms of reconstituted collagen, SLS and FLS, may be interconverted. Extraction of a preparation of FLS with citrate buffer ($\Gamma/2 = 0{\cdot}8$, pH 3·8) and dialysis of the extract against water (pH 4–5) yields a precipitate of SLS. Extraction of a preparation of SLS with citrate buffer ($\Gamma/2 = 0{\cdot}2$, pH 3·8) and dialysis of the extract against water (pH 7) yields a precipitate of material which in form approaches that of FLS.

Further experiments have been carried out as follows:

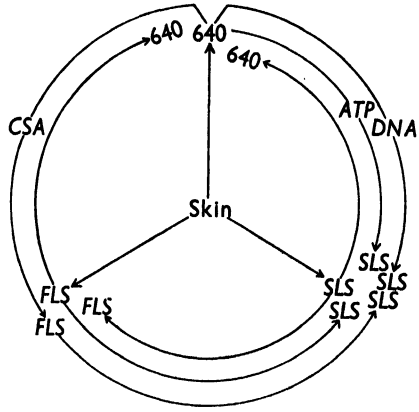
(i) It has been shown in confirmation of the experiments of Schmitt *et al.* (1953) that SLS formed from initial phosphate extracts of connective tissue contain the bases adenine, guanine, cytosine, thymine and uracil. Chromatographic analysis showed these bases to be present in one preparation in the following molar proportions: guanine 27·4%, adenine 22·2%, cytosine 16·2%, uracil 9·0% and thymine 25·2%.

(ii) SLS have been prepared from a rat-skin extract (which contained no nucleotides) by the suitable addition of adenosine triphosphoric acid (0·2%).

(iii) If FLS prepared by the addition of chondroitin sulphate is dissolved in citrate buffer ($\Gamma/2 = 0{\cdot}8$, pH 3·8) and the solution dialysed against water

(pH 4–5) the precipitate contains SLS. This is of interest in that it shows that the SLS type of structure may be obtained by the use of other than nucleotide precipitants.

The results of these and other experiments may conveniently be summarized in a cyclic diagram such as Text-fig. 9, which inevitably oversimplifies the results. It should be understood, for example, in considering the problem of interconvertibility that the yields of the different forms are rarely 100%; material showing fine structure is often associated with material without any structural characteristic visible in electron micrographs.



Text-fig. 9. Cyclic diagram to show the possible methods of interconvertibility of the various structural forms of precipitates. *FLS*, fibrous long spacing; *SLS*, segmented long spacing; *CSA*, chondroitin sulphate; *ATP*, adenosine triphosphoric acid; *DNA*, desoxyribose nucleic acid.

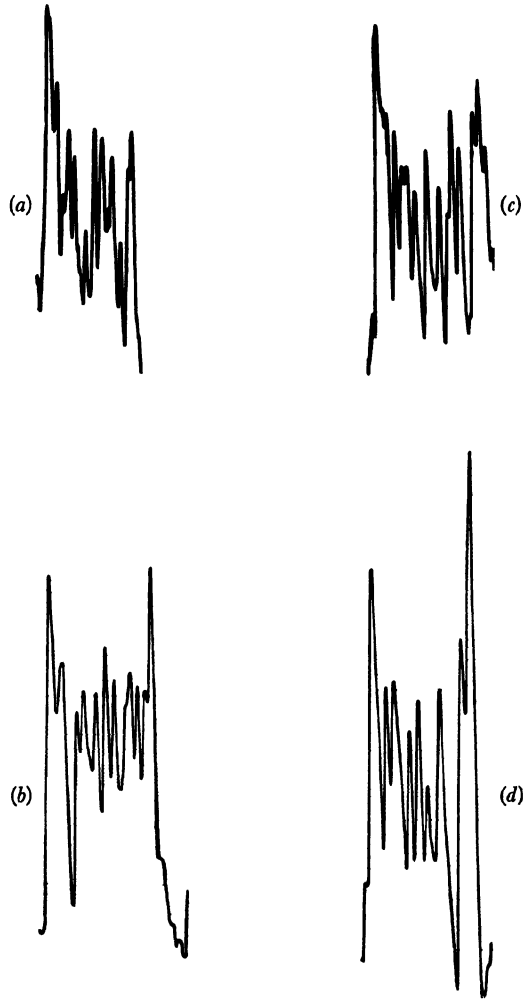
It is of interest to refer briefly to the fine structure observed for the two chief types of complex:

(1) *Fine structure of segmented long-spacing material* (SLS). Our results refer at this date to shadowed specimens only, but we believe this does not affect periodicity measurements. Although segments of a variety of lengths and widths have been observed we do not consider on present evidence that there is any fundamental difference in fine structure between the various specimens examined. All segments referred to in Table 6 are of a polar type which is characterized in Text-fig. 10 by the deep minimum within each period.

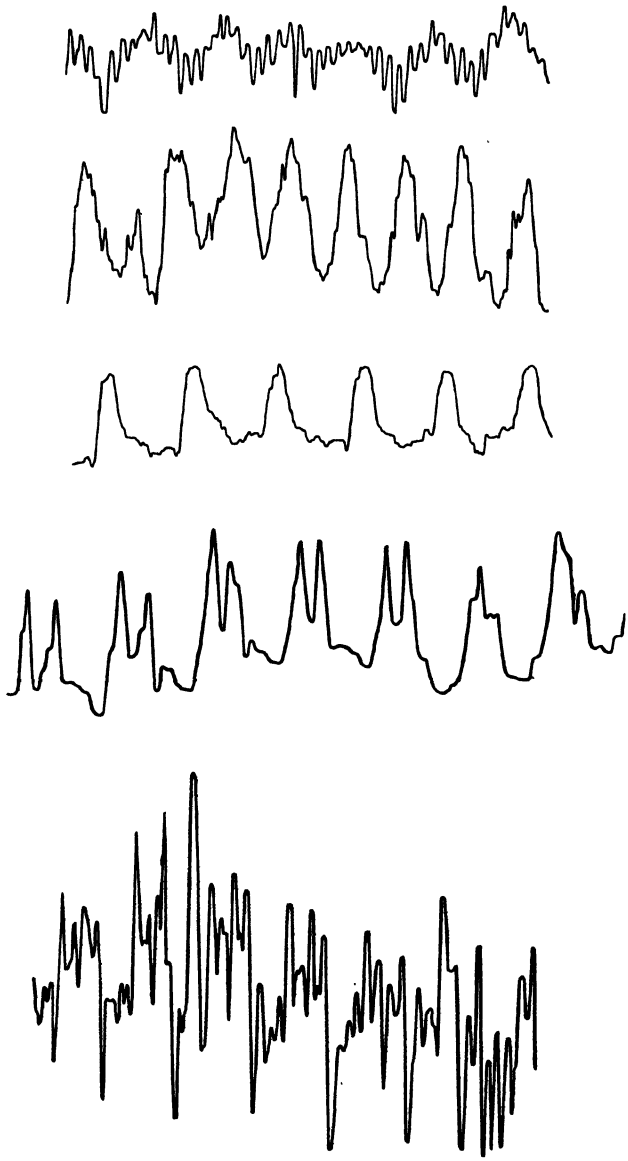
(2) *Fine structure of fibrous long-spacing material* (FLS). It is apparent from a study of our own micrographs and those of Schmitt *et al.* (1953) that there is far greater variety of structure possible in this material, which is illustrated in Text-fig. 11 and which shows the periodicity and fine structure of five different types obtained by the use of various methods in our laboratory.

Table 6. *Comparison of fine structure of segmented long-spacing material*

Method of preparation	Structure in percentage of segment length													
(1) Direct from skin	0	5·4	10·9	24·8	31·6	—	43·4	55·3	66·3	74·8	78·2	84·2	92·5	100
(2) From skin FLS	0	4·5	13·9	24·2	29·2	35·7	44·9	56·2	69·6	76·1	—	83·9	90·9	100
(3) By ATP precipitation	0	—	8·9	—	31·8	—	43·7	53·9	63·0	—	79·0	—	87·4	100
(4) From FLS precipitated by ChS	0	—	10·4	25·6	32·8	—	45·0	54·7	63·2	—	79·0	86·4	94·1	100



Text-fig. 10. Densitometer traces showing the fine structure of segmented long-spacing material obtained by various methods. (*a*) SLS extracted directly from skin; (*b*) SLS obtained from skin FLS; (*c*) SLS obtained by precipitation with ATP; (*d*) SLS obtained from FLS prepared by precipitation with chondroitin sulphate.



Text-fig. 11. Electron density traces of fibrous long-spacing material which show the variations in fine structure of five types of precipitates that are obtained by the different methods in use.

In conclusion the following points should be emphasized:

(i) While it is generally believed that normal fibrous collagen of 640 Å. periodicity arises from a single protein constituent (although it is by no means proved) it is possible that the complex forms of FLS and SLS are formed by the association of a soluble collagen fraction with another molecule usually, but not always, macromolecular.

(ii) Neither fibrous nor segmented long-spacing material has been observed in connective tissues *in vivo*. The demonstration of each type requires certain precise environmental conditions which may not exist in the tissues *per se*; the necessary components are present, but laboratory manipulation is required to produce the FLS and SLS complexes.

(iii) Existing evidence is too meagre to enable us to decide whether the SLS structures are built of *initially* short units of a length ~2000 Å. Schmitt *et al.* (this volume) believe this to be so and have called such units *tropocollagen*. The electron microscope should theoretically be able to distinguish individual polypeptide chains, but in practice the difficulties of doing so are very great. The postulation of such units ~2000 Å. long presupposes that the long chains of the initial solutions are broken down into such short lengths. In view of the discussions in Part II of this paper the possibility that the long chains are first folded into shorter lengths and subsequently interact with each other should not be ignored.

We wish to thank Mr K. L. Hull for his technical assistance in the reproduction of the figures. F.B. acknowledges a grant from the British Empire Cancer Campaign; R.E.B. a maintenance grant from the Department of Scientific and Industrial Research; and F.C.K. a Fellowship from the Nuffield Foundation.

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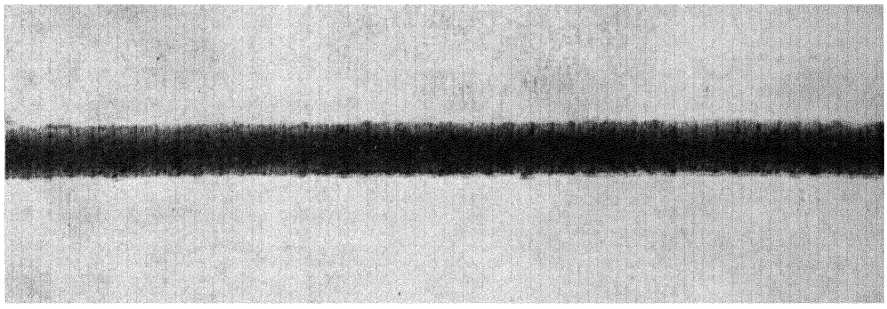
PLATE I



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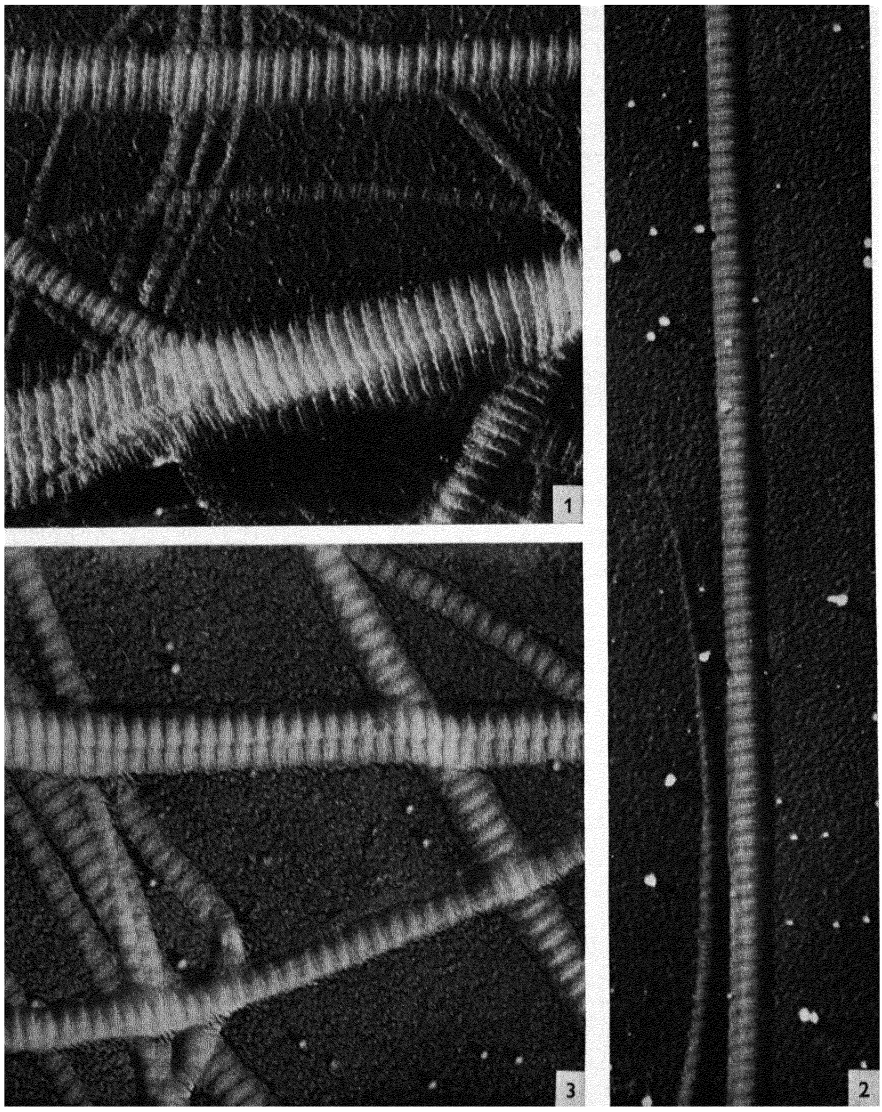

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EXPLANATION OF PLATES

PLATE 1

Electron micrograph of unstained, unshadowed native collagen from fowl-neck tendon; the true variations in density of the band structure within the main period of the fibre can be seen. × 50,000.

PLATE 2

Fig. 1. Electron micrograph of collagen fibres precipitated from an 0·006% solution of collagen in 0·01 M-acetic acid by the addition of sodium acetate to give an initial pH of 5·8 followed by dialysis against water of pH 7·5. Shadowed Au Pd. × 40,000.

Fig. 2. Electron micrograph of collagen fibres obtained by dialysis of an 0·002% solution of rat-tail collagen in 0·001 M-sulphuric acid against conductivity water of pH 7·0. Shadowed Au Pd. × 25,000.

Fig. 3. Electron micrograph of collagen fibres obtained by dialysis of an 0·018% solution of ichthyocol in 0·01 M-acetic acid against conductivity water of pH 7·0. Shadowed Au Pd. × 25,000.

STATES OF AGGREGATION OF COLLAGEN*

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This paper is intended primarily to indicate results obtained by the authors during the last few years on the structure, composition and states of aggregation of collagen particles as they apparently occur in certain types of solutions. During this period several papers have appeared (Highberger, Gross & Schmitt, 1950, 1951; Gross, Highberger & Schmitt, 1952; Schmitt, Gross & Highberger, 1953; Gross, Highberger & Schmitt, 1954) which were concerned primarily with a description of the 'long spacing' (LS) forms of collagen, both fibrous and segmental, and of conditions under which these forms could be produced. In this paper we propose to discuss the structure and composition of three forms of collagen—standard fibrous collagen, fibrous long-spacing (FLS) and segment long-spacing (SLS)—factors which determine the interconvertibility of these forms and, finally, the possible bearing of these facts on the existence of a type of particle, herein given the name 'tropocollagen'. Briefly, this particle is considered to be a kinetic unit of collagen, consisting of very thin, elongated particles having lengths equal to the long spacings and being capable of reversible conversion into fibrous collagen, FLS or SLS.

Major problems in this field include: (1) the detailed structure of polypeptide chains in collagen, as deduced primarily from X-ray diffraction, and the sequence of amino-acids along the chains; (2) the structural and chemical significance of the axial pattern of banding in collagen, FLS and SLS; (3) the properties of a type of collagen particle, *tropocollagen*, and its relation to the collagenous structures thus far described; and finally (4) the biological mechanisms by which collagen is synthesized and built into the fibrous structure of connective tissue. In the following we shall be concerned primarily with items (2) and (3) above.

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I. CHEMICAL COMPOSITION OF PURIFIED HIDE COLLAGEN

Studies of the composition of purified collagen are not lacking (see particularly Bowes & Kenten, 1948; and Neuman, 1949). Although the chemical analytical methods have in general been satisfactory, in no case have self-consistent criteria of purity been set up nor have exhaustive attempts been made to achieve such purity.

Hide, obtained from an old (13 years) cow, was used in the present experiments because such material has a minimum of ground substance. The purification consisted of extensive extractions with 10% NaCl followed by extractions with 0·15 M-Na_2HPO_4 after the method of Bergmann & Stein (1939). In each case extractions were continued until no further protein was removed.* After dialysis against cold water, to remove all phosphate, the purified collagen was subjected to repeated extraction with half-saturated $Ca(OH)_2$. The extraction was continued until it was demonstrated analytically that the material being extracted was similar chemically to that of the residue. At this point it was assumed that, within the accuracy of the analytical methods, all material not originally present in the organized collagen fibrils had been removed; hydroxy proline and glycine were present in the same proportions in extract and residue. The sample, which originally weighed 25 g., was subjected to eleven extractions with NaCl, nine with Na_2HPO_4 and sixty-two with half-saturated $Ca(OH)_2$.

It was found that hexose is still present in appreciable quantity after these treatments ($\sim$0·4%). Galactose and glucose appear to predominate in the final product, but mannose was also present in many of the $Ca(OH)_2$ extracts. The final product of these treatments also still contains hexosamine, but the amount is very small (0·05%).

It has long been known that a small ($<$1%) carbohydrate moiety is closely associated with collagen. It is not known whether the carbohydrate here demonstrated to occur in the most highly purified collagen exists as individual molecules, as part of a polysaccharide or as part of the collagen structure itself. It has been suggested (see Meyer, 1946) that substances such as chondroitin sulphate or hyaluronic acid are involved in the biogenesis of collagen (as a fibrous lattice on which the soluble collagen condenses). However, there is no direct evidence that mucopolysaccharides are structurally significant components of the collagen molecule or are

* Extensive extraction with NaCl removed of the order of 1% of the total dry protein present in the corium. Phosphate extraction removed about 0·2% on the same basis. Obviously the standard Bergmann procedure is not sufficient to remove completely all non-collagenous material. Extraction with half-saturated lime solution removed about 17% of the protein.

involved in its biogenesis. Jackson (1953, 1954) has claimed that chondroitin sulphate forms stabilizing linkages between collagen chains, based primarily on his observation that crude hyaluronidase increases the acid solubility of collagen. The highly purified collagen described above shows no enhanced acid solubility although the amount of chondroitin sulphate that could be present (even if the total hexosamine came from this source) would be so small that it is difficult to see how it could play such a role; there is one hexosamine molecule per 360,000 molecular weight of protein. In addition we have found (unpublished) that proteolytic enzymes such as papain and erepsin also increase the acid solubility of various collagens. It should be emphasized that the precipitation of collagen fibrils by various substances is no proof that these substances form an essential part of the structure of native collagen or are involved in its biogenesis.

The tyrosine content, though greatly reduced from that of the original corium, is still appreciable (0·41 %) in the most highly purified collagen which we have prepared. Orekhovitch, Tustanovsky, Orekhovich & Plotnikova (1948) and Orekhovitch (1952) claim that their samples of procollagen are completely lacking in tyrosine. In large series of experiments we have tried to obtain such tyrosine-free collagen. In a few instances values somewhat lower than the 0·41 % here reported were obtained but we have never got a preparation which gave a negative Millon reaction.*

The collagen residue was examined in the electron microscope from time to time as the lime extraction proceeded. The residue showed the characteristic banded appearance even after sixty-two extractions. Of course at that time some of the collagen fibrils were being broken down and were passing into the extract. Electron-microscopic examination might therefore be expected to show a certain amount of amorphous material and broken-down fibrils, as was in fact the case. The results of these analyses leave open the question whether the non-protein, and perhaps the aromatic amino-acid constituents, are part of the collagen 'molecule'.

II. THE FINE STRUCTURE OF COLLAGEN FIBRILS

With improvements in technique and resolution it has been possible over the years to demonstrate an increasing degree of fine structure of collagen fibrils as seen with the electron microscope. Originally only one dark and one light band per period were observed (Schmitt, Hall & Jakus, 1942; Wolpers, 1943). Then two or three (Wolpers, 1944; Hall, Jakus and Schmitt, 1946), five (Schmitt *et al.* 1945), six or seven (Nutting & Borasky, 1948;

* For the quantitative Millon reaction used in our experiments the method of Bernhart was employed, as outlined by Block & Bolling (1951).

Schmitt & Gross, 1948; Wolpers, 1949, 1950), and eventually ten (Hofmann, Nemetschek & Grassmann, 1952) intraperiod bands were observed. The band pattern is polarized, i.e. asymmetric.

These bands have characteristic intraperiod position and density in preparations stained with phosphotungstic acid; they presumably reflect both a constancy of chemical composition and a regularity of structure of the collagen fibrils. So far as is known the pattern is unique for collagen.

Presumably the bands represent variations in the structural interaction of adjacent polypeptide chains at the various levels within each repeating period. It has been suggested that when the discontinuities in adjacent chains are maximally in register the fibrils show the greatest number of intraperiod bands. If the discontinuities were completely out of register, i.e. randomly arranged in adjacent chains, the fibrils might conceivably show no repeating period at all; fibrils formed by the action of 5% NaCl on an acid solution of ichthyocol collagen usually show no bands. Wolpers (1950), on the other hand, believes that normal native collagen fibrils show only two bands per period; when more bands are seen, this is regarded by him as a pathological manifestation.

It is significant that frequently only two bands per period are observed, although the resolution may be entirely adequate to observe ten bands if they did, in fact, exist. In such cases the structure may be essentially unpolarized. Indeed, the fact that the axial period averages about 640 Å. may be about the only evidence that such material is collagen.*

It has been assumed by us that in collagen fibrils showing the full characteristic polarized intraperiod banding, the collagen protofibrils† pursue an essentially parallel‡ course through the fibril; the banded structure therefore represents discontinuities existing in the protofibrils themselves. When fibrils known to be collagenous show only one or two bands per period and the structure is essentially unpolarized, it may be that the collagen chains pursue an antiparallel course, i.e. that the fibrils contain mixtures of chains running in the two directions. It is also possible that such fibrils contain, in addition to the polarized protofibrils of indefinite

* If the material is of such a nature that it is impossible to subject it to chemical or X-ray analysis, one must be very cautious, on the electron-microscope evidence alone, to make the identification. For example, intracellular fibrils (ciliary rootlets of Fawcett & Porter, 1954) may show a clear-cut banded structure with a period near to that of collagen. However, unless characteristic intraperiod fine structure of collagen can be shown, it would be premature to conclude that the material is collagenous. It is quite possible that other proteins may have an axial repeating period near to 640 Å.

† The term protofibril is here used essentially in the sense originally defined by Schmitt *et al.* (1942) as the elementary fibrous particle which gives rise to the large-angle X-ray pattern of collagen. Whether the protofibril is composed of one or more than one polypeptide chain in a particular helical configuration remains to be seen.

‡ The terms parallel and antiparallel are here used to denote situations in which the structurally polarized chains are all pointing in the same direction (parallel) or in mutually opposite directions (antiparallel).

length, also short particles (tropocollagen); such an admixture might show any type of fine structure from that characteristic of collagen to a simple one-banded structure or any intermediate form such as that shown in Pl. 2, fig. 1 *d*, which appears to depict a modulation of a two-banded collagen structure by FLS (accentuation of every fourth band pair).

Particularly to those investigating the biogenesis of collagen it is important to realize that collagen (possibly primarily in the form of tropocollagen) may exist outside the well-organized fibrils, either in the ground substance or in cells. Probably the collagen which can be extracted by the mildest means (salt solutions at or near neutral pH) contains tropocollagen in the polymerized and in the unpolymerized form. The high viscosity of such extracts suggests that the equilibrium is pushed far in the direction of polymerization (Gross *et al.* 1954).

III. STRUCTURE, COMPOSITION AND FORMATION OF FIBROUS AND SEGMENTAL LONG SPACINGS

As previously shown (Highberger *et al.* 1951; Schmitt *et al.* 1953), both FLS and SLS may be observed in extracts of connective tissue without the addition of extraneous material. For example, FLS was originally observed in citrate extracts after dialysis against water ('procollagen'). SLS was observed in mildly alkaline phosphate extracts after dialysis against acid citrate buffer. In each case it was supposed that the long-spacing form was produced because there was present, in addition to the soluble collagen, another component which induced the formation of LS material.

In the case of FLS it was at first supposed that the inducing agent was acid glycoprotein (Highberger *et al.* 1951). However, the conclusion was reached (Gross *et al.* 1952) that the structural specificity resides in the collagen itself rather than in the inducing agents, because we could show that FLS can be produced by the addition of a variety of substances, including chondroitin sulphate, thrombin, heparin, etc. (see also Randall, Fraser, Jackson, Martin & North, 1952). A similar experience was recorded in the case of SLS. The observation that phosphate extracts characteristically showed an absorption maximum at about 2600 Å. suggested that nucleic acid or nucleotides might be required for the production of SLS. Working on this hypothesis, the discovery was made that SLS could be produced at will by the addition of adenosine triphosphoric acid (ATP) to filtered acid solutions of collagen. However, it was possible to show that SLS could be produced in the apparent absence of material absorbing at 2600 Å.,* again

* For example, LS may be induced by materials, such as chondroitin sulphate, which do not absorb in the region of 2600 Å.

suggesting that the specificity resides in the collagen rather than in the inducing agents.

SLS could be formed from mildly alkaline phosphate extracts of *purified* collagen by dialysis against acid citrate only when substances such as nucleic acids, chondroitin sulphate or heparin were added. The amount of SLS precipitated in such experiments varies with the concentration of added agent. The added substances may function by association of collagen particles through charged groups on the inducing agent. This speculation will be considered in more detail below, but it may be desirable first to examine the structure of FLS and SLS; a more detailed description is given by Gross *et al.* (1954).

A. *Fine structure of fibrous long spacing*

In Pl. 2, fig. 1, are shown several characteristic types of FLS obtained on dialysis of citrate extracts of connective tissue and of acetic acid solutions of ichthyocol (in the latter case after the addition of α-1 acid glycoprotein). Pl. 1 shows a schematic diagram representing the band structure of FLS. The nomenclature of the bands is entirely arbitrary and should be considered provisional.* If any clear-cut relation could be adduced between any particular bands observed in collagen fibrils and those seen in FLS, appropriate nomenclature could be adopted. For the present we shall assume: (*a*) that FLS fibrils are composed of very thin elongate particles having lengths approximately equal to the long spacing; (*b*) that the particles are in *antiparallel* array, giving rise to the non-polarized, symmetrical structure; (*c*) that the particles are composed of collagen (possibly in association with a variable amount of non-collagenous material). In support of the last point it may be mentioned that the amino-acid composition of FLS is, qualitatively at least, similar to that of collagen. Also, the large-angle X-ray diffraction pattern of purified preparations of FLS (taken with the beam parallel with the surface of a film) is that characteristic of collagen.

The fine structure of FLS may vary considerably (see Pl. 2, figs. 1 *a*–1 *d*). As in the case of collagen, this variability may conceivably be due to an out-of-register effect in neighbouring tropocollagen particles or chains of particles and possibly to an admixture of collagen and tropocollagen with the FLS.

In a few isolated cases there were observed (Highberger *et al.* 1950), in dialysed citrate extracts ('procollagen'), individual segments having the symmetrical, FLS structure.

* Bands seen in FLS will be represented as *fa*, *fb*, *fc*, etc. If, as seems likely, subsequent research reveals further fine structure, this may be denoted as *fa*-1, *fa*-2, etc. Similarly, the bands in SLS will be denoted as *sa*, *sb*, *sc*, etc. We should like to suggest that the bands in collagen be denoted *ca*, *cb*, *cc*, etc. This implies no necessary correspondence in structure or composition between *ca*, *fa* and *sa*, between *cb*, *fb* and *sb*, etc.

B. *Fine structure of segment long spacings*

In Pl. 2, figs. 2, 3 and Pl. 4, figs. 2, 3, are shown some characteristic electron micrographs of SLS produced by addition of ATP to ichthyocol acid filtrates. A schematic representation of the major bands observed is given in Pl. 1. As many as twenty-five bands have been seen in individual segments, but only the major bands and band complexes are shown in Pl. 1.

The striking characteristics of SLS are: (*a*) the long spacings occur as segments usually quite sharply defined; (*b*) the structure is very clearly asymmetric (this is most strikingly indicated by the fact that the interband of low density between the *sf* and *sg* bands, shown by the arrows in Pl. 2, fig. 2, is usually very distinctly off centre); and (*c*) the amino-acid composition and large-angle X-ray diffraction pattern of SLS are similar to those of collagen.

As previously shown (Schmitt *et al.* 1953) the distribution curve of segment lengths is rather broad, from about 1500 to 3000 Å. However, despite variations in lengths of segments there is fairly close correspondence in the absolute positions of the bands in the region from the *sc* to the *sh* bands (see Pl. 1). It is possible that the filamentous particles heap up at the ends of the segments causing the thickness to be greater there (as shown in shadowed preparations) and contributing to the variation in length of the segments.

It is possible by superposition of the band structure of SLS upon itself, with the polarization of the two patterns made opposite (antiparallel), and with a shift from coincidence of the ends of the patterns, to obtain a resultant symmetrical pattern resembling that of the repeating unit of FLS. It is obvious that the above kind of superposition in opposite polarities must result in a symmetrical, unpolarized structure. The fact that to obtain a structure resembling that of FLS requires shifting the SLS patterns somewhat along the axis may be significant from the structural standpoint. Such matters will be discussed more fully in a subsequent paper dealing with the fine structure of collagen, FLS and SLS.

C. *Mode of formation of FLS and SLS*

Much has been learned by studies of systems which are in the process of forming FLS, SLS or collagen, and further studies along these lines are in progress. In such 'time-sequence' experiments, samples are removed at specified intervals of time and their contents examined with the electron microscope. Obviously many samples would be required to give statistical significance to conclusions reached. However, in the present paper we shall indicate the story which may be told in a qualitative manner.

In time-sequence studies of FLS formation, glycoprotein was added to a filtered acid solution of ichthyocol and the mixture dialysed. Soon after the beginning of dialysis characteristic, small, very dense 'spheroids' are observed which have diameters in the range of 400–800 Å.* When the concentrations of glycoprotein and of collagen are sufficiently high, these spheroids appear to be associated with the laying down of FLS. Although little is known about the details of this process, we suspect that the dense particles consist of glycoprotein to which, by co-ordination around its many negative charges, collagen filaments have become anchored. Frequently, characteristic 'spindles' are observed (Pl. 3, fig. 2) which may be the precursors of FLS as dialysis proceeds. We should like to suggest that these spindles represent tactoidal aggregates of tropocollagen particles anchored at their ends by glycoprotein. When these aggregate, a tenuous gel is formed from which FLS emerge.

It was early observed that FLS fibrils form from large cloud-like, tenuous gelled aggregates as the mixture of ichthyocol acid filtrate and glycoprotein dialyses. Time-sequence studies suggest that fibrils form from the 'matrix' of the cloud probably by a condensation of fibrous structures.† This is illustrated in Pl. 3, fig. 1, which suggests that the dense clouds consist of fairly close, essentially hexagonally packed areas whose centres are the dense particles and whose separation, centre to centre, is approximately that of the LS period.

A variant of this process of FLS formation was observed with different collagen solutions as follows: dense spheroids were observed as with the other preparations, but the next step did not involve the condensation of cloud-like networks but rather the formation of thin 'long periods' having a dense spheroid at either end. A few minutes later there appeared short, linear aggregates of two to six or more periods, together with tactoidal and fibrous FLS (Pl. 3, fig. 2).

In the formation of SLS by the action of ATP on ichthyocol acid filtrate, dense spheres were observed to form immediately after the mixing of ATP and filtrate (Pl. 4, fig. 1). These spheres appear to be involved in the formation of SLS; they may be observed in large quantities during the formation of SLS but are not present when the concentration ratios of ATP and collagen are such that SLS form very rapidly. Apparently after the SLS are fully formed, no dense particles are seen because they have then been

* The spheroids, here implicated in the formation of FLS, have dimensions of the same order as those of the globular particles (640 Å.) which Randall (1953) suggested might form the collagen fibril. We have no evidence that the spheroids actually form part of the collagen fibrils, though they might well be involved in the formation of the fibrils.

† Dense spheroids have occasionally been observed to form in the early stages of the dialysis of ichthyocol acid filtrates against 1 % NaCl. Conceivably the collagen particles in this case aggregate around salt-precipitated nuclei.

used up in the process. We suspect that the dense spheroids consist primarily of ATP molecules which have bound collagen particles through their negative charges. These particles, by a process of self-ordering, form SLS.

D. *Interconvertibility of fibrous collagen, FLS and SLS*

Schmitt *et al.* (1953) indicated ways by which it is possible to interconvert these forms apparently reversibly. At that time the evidence was essentially electron microscopic; after conversion the sample appeared to contain almost exclusively the converted form. This is not conclusive evidence because the electron microscope is a notoriously poor sampling device (due to the high magnifications used) and because a substantial fraction of the sample may have been extremely thin filaments or particles likely to escape detection unless special precautions are used. Gravimetric evidence, presently in hand, while still not constituting final proof, is consistent with interconvertibility. By this means (and using hydroxy proline and glycine analyses as criteria of collagen content) we have demonstrated a 50–75% conversion of one form into another. We believe that the true figure is actually substantially higher because of the loss, which has so far proved unavoidable, of material on the walls of dialysis bags and in lyophilization. Since it was not possible to recover control material after dialysis and lyophilization to an extent greater than 75%, the interconversions may have been 75–100% complete. At any rate it was shown, in particular experiments, that there was little collagen in supernates of preparations whose sediments were judged to consist almost entirely of the converted form.

IV. TROPOCOLLAGEN AND ITS RELATION TO OTHER FORMS OF COLLAGEN

Discovery of the FLS form of collagen (Highberger *et al.* 1950) soon led to the view that the long spacing reflects the presence of thin *particles* having lengths equal to the axial periods and arranged in a particular type of intrafibrillar packing. In the spring of 1952 our attention was focused on the isolation of these particles which we came to regard as the unit particles of which collagen is made. It was felt that only when this material was in hand in purified form could one succeed in making substantial advance in determining the chemical composition, structure and eventually the biogenesis of collagen. At that time we adopted the term 'tropocollagen' to denote the 'monomer' particles which, when aggregated in a particular manner, produced collagen. The word collagen is etymologically ill conceived from the standpoint of descriptive protein chemistry. One could hardly add the

suffix 'gen' to it to indicate its precursor as is frequently done in protein chemistry. Since the words *procollagen* and *precollagen* have already been pre-empted, we decided to adopt the term *tropocollagen.**

If tropocollagen occurs in native tissues it should presumably be present in the ground substance rather than in the organized fibrils and should be soluble in salt solutions at near neutral reaction. Accordingly, extracts were made with phosphate buffer and with salt solutions of skin and swim-bladder tunic (Gross *et al.* 1954, 1955). Dialysis of such extracts against water leads usually to an amorphous precipitate.† However, when dialysed against citrate buffer, pH 4, a precipitate was produced which contained the previously undiscovered SLS.‡ Because phosphate extracts frequently showed an absorption maximum at 2600 Å., it was supposed that nucleotides or nucleic acid might be involved, and this led to the discovery that SLS may be produced directly from acid extracts of collagen by the addition of ATP.

These discoveries were consistent with the original tropocollagen hypothesis. Under the influence of ATP, possibly because the tropocollagen particles were arranged in parallel rather than in antiparallel array, axial aggregation did not occur; segments rather than fibrils were formed. Strong evidence in support of the hypothesized tropocollagen particles are the fray pictures obtained from preparations of SLS in acetic acid. In the absence of ATP, acetic acid causes the SLS slowly to decompose into their constituent particles and, especially in the presence of salt, to form fibrous collagen. Shadowed pictures of the frayed segments show these particles, which are probably bundles of a few tropocollagen particles (Pl. 2, fig. 3).

The marked tendency for tropocollagen particles to aggregate made isolation and characterization of the material difficult. However, discovery of this tendency led to the experiments which demonstrated the interconvertibility of tropocollagen, collagen, FLS and SLS as described above. It should perhaps be emphasized that these experiments were performed with purified solutions *in vitro*. It is entirely possible that, in the organism, the conversion of tropocollagen into fibrous collagen is not fully reversible but may involve chemical alterations, such as occur when fibrinogen is converted to fibrin, with the splitting off of a fragment, fibrinopeptide (Lorand, 1952).

* The combining form Tropo, from the Greek *Tropos* meaning a turn or change, i.e. to turn into or change to collagen.

† In some cases, however, such dialysis leads to the formation of FLS.

‡ Segments were actually observed along with FLS in citrate extracts after dialysis (Highberger *et al.* 1950), but these had fine structure different from that of the asymmetric, polarized SLS. Also they were seen only sparsely in the earlier preparations and seldom if at all, in later preparations.

In any particular collagenous system it may be useful to consider that an equilibrium exists between the free tropocollagen particle and the highly polymerized fibrous form. Under certain conditions, such as in most adult tissues, probably because of the relatively high ionic strength, the equilibrium is pushed far in the direction of high polymerization. Although no cases of collagen long spacings have thus far been observed in native material, it is entirely possible that conditions might, in certain cases, permit this type of aggregation of tropocollagen rather than the usual fibrous collagen type.

If collagen is formed by linear and lateral aggregation of tropocollagen particles the question may be asked: What is the significance of the 640 Å. axial period and of the detailed pattern of intraperiod banding seen in the electron microscope? Several possibilities, though highly speculative, might be suggested. The bands in collagen, as well as in SLS, FLS and other regularly cross-striated fibrous proteins, may be due to interaction of neighbouring protofibrils, through side-chains to form regions of relative order and disorder, as suggested by Bear (1952). This regularity, in turn, depends on a regularly repeated sequence of amino-acid types if not of the residues themselves. When collagen protofibrils (presumably consisting of an adlineation of tropocollagen particles) aggregate laterally under appropriate conditions the repeating pattern is that of native collagen. When the protofibrils are in antiparallel array, the usual band pattern is that characteristic of FLS. When the same particles with the same sequence of amino-acid residues are aligned in parallel array, presumably with a different matching of side-chain partners than that characteristic of collagen, the tendency for lateral aggregation is strong while that for linear aggregation is very weak and SLS are formed. In this sense the 640 Å. period (700 Å. in the moist state) would not necessarily represent a molecular domain any more than does the long spacing, three to five times this length. Such a view is, of course, consistent with the possibility that the tropocollagen particle may be composed of one or more species of subparticles (such as a 640 Å. particle as suggested by Randall, 1953) which may conceivably be capable of being demonstrated by appropriate chemical procedures.

The tropocollagen-collagen system bears some resemblance to the fibrinogen-fibrin system. Fibrin has a very regular axial repeating period, as manifested in the banded pattern seen in the electron microscope. This period (about 230 Å.) bears no obvious relation either to the length of the fibrinogen molecules (about 500 Å.) or to the subparticles which have been resolved in the electron microscope (Hall, 1949; Siegel, Mernan & Scheraga, 1953). According to Ferry, Katz & Tinoco (1954) fibrin forms from the polymerization of fibrinogen through an intermediate polymer step. The

intermediate polymer has a particle weight of $5{\cdot}6 \times 10^6$, a length of about 3200 Å. and is pictured as a double row of staggered fibrinogen molecules 6 to 9 per row, with a polymerization number of 15. These intermediates are thought to be in a reversible equilibrium with the activated monomers. It was suggested that the band pattern with axial period of 230 Å. might result from lateral interaction of neighbouring intermediate polymers or fibrin protofibrils. If tropocollagen corresponds in this analogy with the activated monomer molecules of fibrinogen the axial repeating period of collagen would, like that of fibrin, be smaller than the length of the monomer particle though the ratio in the two cases would, of course, be different. If it should eventually be found that particles corresponding to the 640 Å. units (possibly the 'molecular' units of collagen) exist as kinetic units in solution then tropocollagen would probably represent aggregates of several of these units stable under the conditions of the *in vitro* experiments here described. Under these conditions tropocollagen would correspond to the intermediate polymers (rather than fibrinogen) in the fibrinogen-fibrin system.

When an acid solution of collagen (ichthyocol) is carefully warmed, certain labile bonds are apparently broken which lead to the formation of a material in monodisperse form, having a particle weight of about 70,000 (Gallop, 1953). This material was called 'parent gelatin', after the term used by Scatchard, Oncley, Williams & Brown (1944). It has a low optical activity and is thought to be a configurationally disturbed product of collagen. Being a gelatin this material cannot be reconstituted into collagen. Parent gelatin appears to represent a reproducible unit degradation product of collagen (hence presumably also of tropocollagen), which may be a manifestation of a subunit of collagen (or tropocollagen). Until tropocollagen has been isolated and characterized with respect to particle weight, dimensions and other properties, it will be impossible to determine more closely the relation between tropocollagen and parent gelatin.

It is important to distinguish carefully between procollagen and tropocollagen. Procollagen is the name given by the Russian investigators (Orekhovitch *et al.* 1950) to the collagenous material obtained from acid citrate extracts of connective tissue. After dialysis against water several types of fibrous structures are formed (Highberger, Gross and Schmitt, 1950). The suggestion, implicit in the name, that procollagen is the biological precursor of collagen is rendered highly improbable by the finding of Harkness, Marko, Muir and Neuberger (1954) that isotopically labelled glycine is incorporated only very slowly in the acid citrate extractable fraction. Moreover a portion at least of the collagen extracted by acid citrate is derived from the formed fibrils of the corium because electron

microscope examination of the extracted residue shows considerable disorganization of the fibrils. If the collagen chains in native fibrils be considered to be of indefinite length it would appear that acid citrate buffer disrupts these protofibrils into lengths of some tens of thousands of Angstrom units. The extracted fibrous particles, according to McEwen and Pratt (1953) and Gallop (1953), have lengths of this order of magnitude and weights in the range of 10^6 to 10^7.

Tropocollagen, on the other hand, is the term which we have applied to the monomeric unit of which we assume all collagenous structures to be composed. It has a length of about 2000 Å. and is thought to contain only one or a few polypeptide chains in cross-section. No monodisperse preparations of these particles have as yet been obtained; this is in part due to strong end-to-end and lateral interaction of the particles, which leads to the formation of fibrous structures. The nearest we have come to visualizing the tropocollagen particles in the electron microscope is in the tactoidal aggregates (Pl. 3, fig. 2) observed in the time-sequence studies of the formation of FLS and in the acid disintegration of SLS (Pl. 2, fig. 3). The SLS themselves may be thought of as small 'microcrystals' of tropocollagen.

It is supposed that the protofibrils of native collagen (or of extracted 'procollagen') consist of an adlineation of tropocollagen particles. The inter-particle bonding must be relatively weak because substantially all of the collagen in acetic acid solutions of ichthyocol can be converted into SLS by the addition of ATP.

The tropocollagen hypothesis supposes that this particle is the biological precursor of collagen, synthesized by the connective tissue cells and aggregated into collagen fibrils in extracellular space. When definitive procedures have been developed for the isolation and determination of tropocollagen it will become possible to study the biosynthesis of this substance, its transformation into fibrous collagen and the various intermediate biochemical steps, some of which (like the blood-clotting system) may involve enzymes and other factors.

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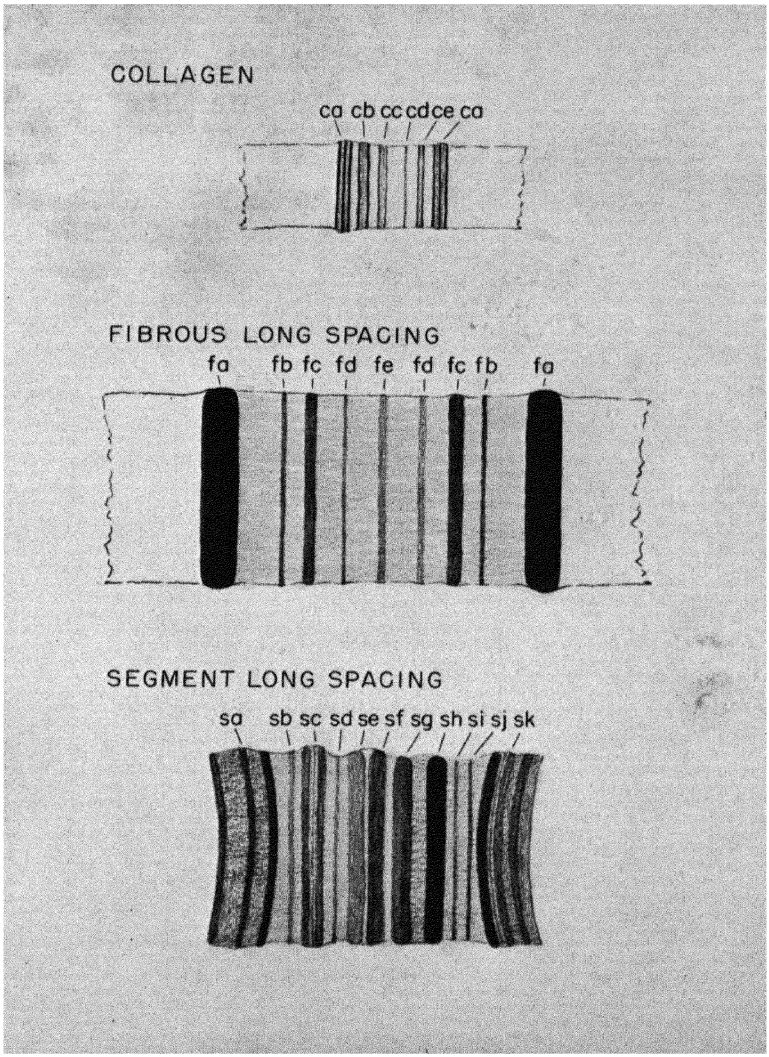
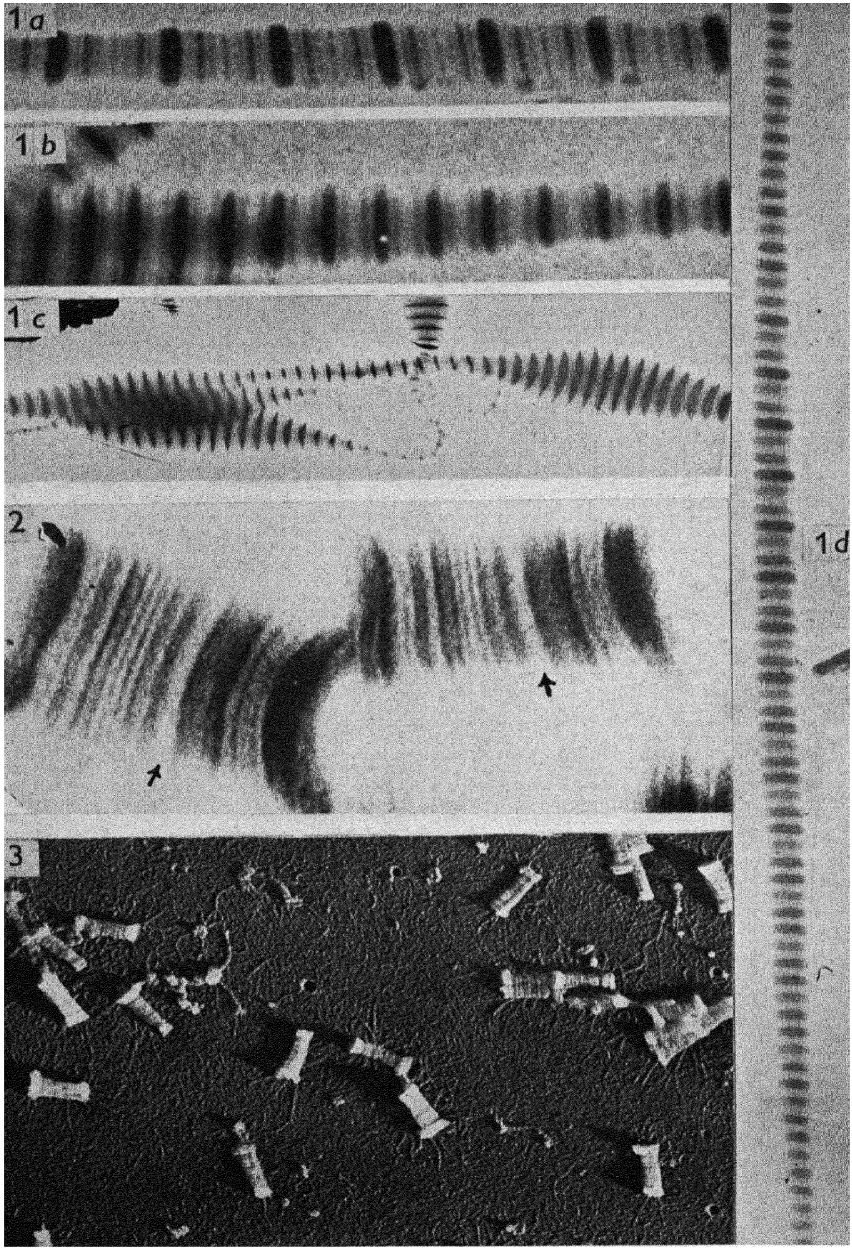
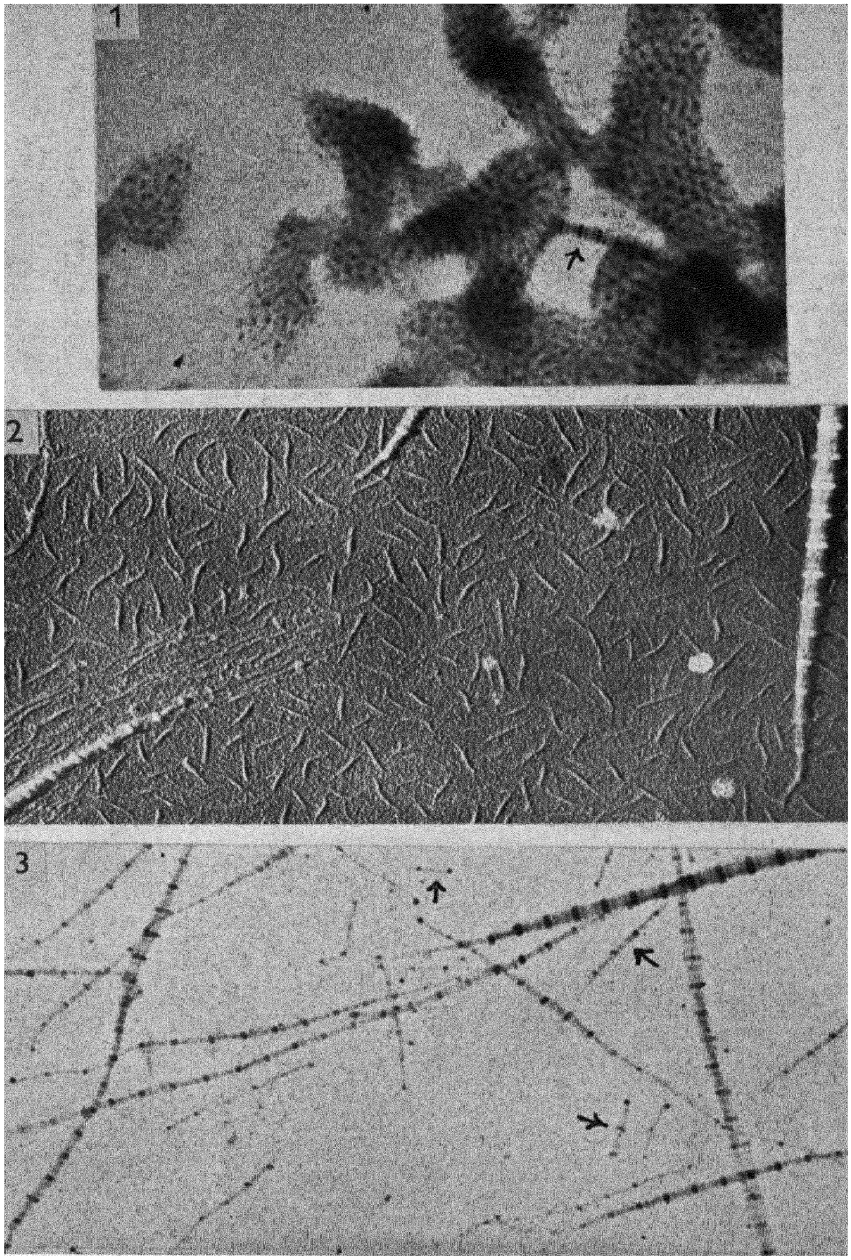


For explanation see p. 162

(*Facing p.* 160)



For explanation see p. 162



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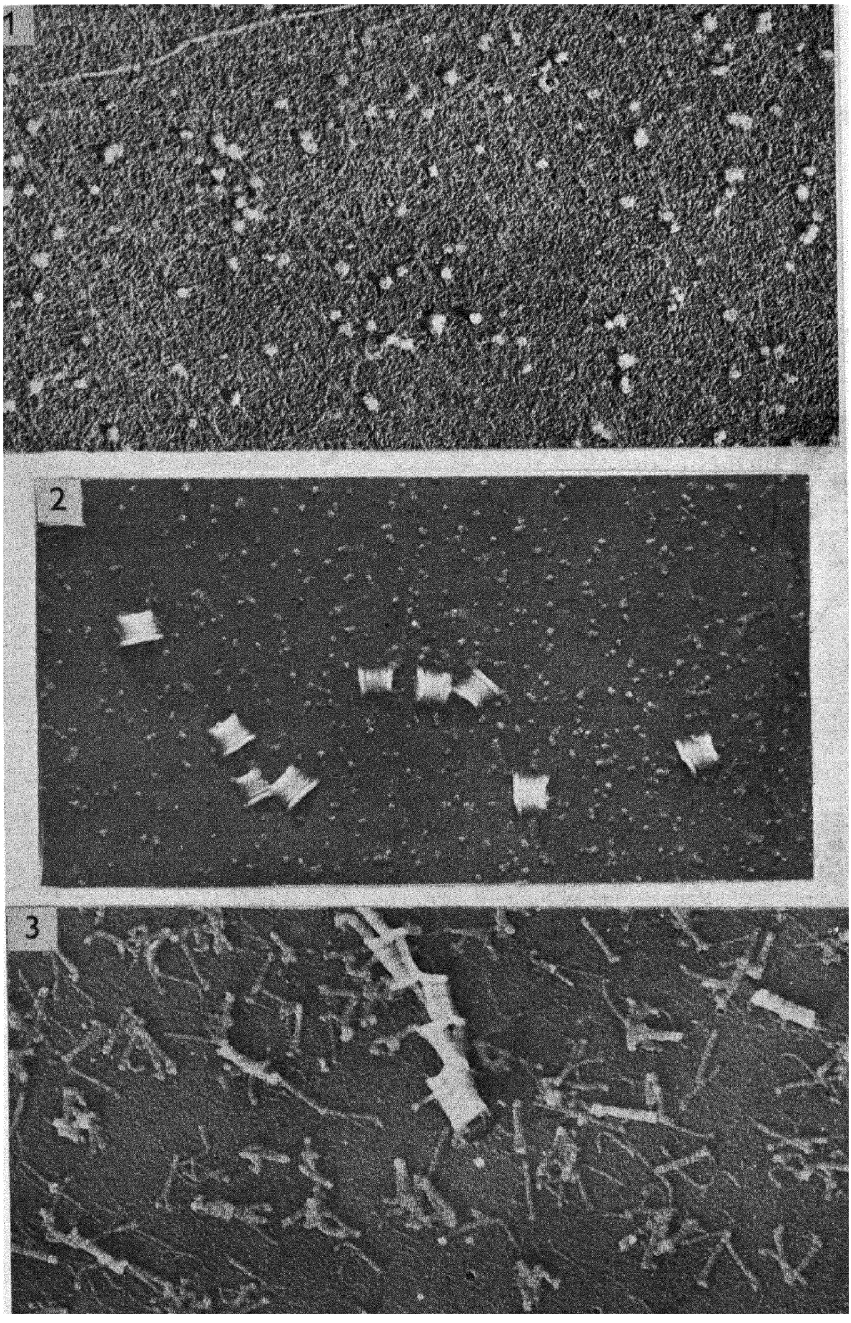

For explanation see p. 162

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EXPLANATION OF PLATES

PLATE 1

Schematic representation of the fine structure of collagen, fibrous and segment long spacing. Stained with phosphotungstic acid. Magnification about 200,000.

PLATE 2

Fig. 1. Types of FLS obtained by dialysing a mixture of ichthyocol acid filtrate and α-1 acid glycoprotein (0·06 %). Difference between types presumably due to differences in the ichthyocol acid filtrates and in the conditions of the experiments.

a. Showing detailed intraperiod structure. Magnification × 68,000.
b. Three-banded structure. Magnification × 32,000.
c. Tactoidal type. Note apparent tilting of major bands. Magnification × 9000.
d. Intermediate form. Note that double-banded collagen structure is modulated in centre of fibril by intensification of every fourth pair of bands, forming a pseudo long spacing. Magnification × 35,000.

Fig. 2. Segment long spacing produced by the action of adenosine triphosphate (0·2 %) on a filtered acid solution of ichthyocol. Stained with phosphotungstic acid. Arrows point to the *sf–sg* interband of low density. Magnification × 180,000.

Fig. 3. ATP-produced segments washed in acetic acid. Shadowed with chromium. Note fraying of segments into very thin, filamentous components (thought to be composed of a parallel packing of tropocollagen particles). Magnification × 28,000.

PLATE 3

Stages in the formation of FLS by dialysis of a mixture of filtered 0·05 % acetic acid and 0·04 % α-1 acid glycoprotein (Schmitt).

Fig. 1. Sample taken after 45 minutes of dialysis. Stained with phosphotungstic acid. Note condensed 'clouds' with dense spheroids regularly arrayed in some places. Also emerging LS fibril (arrow). Magnification × 12,000.

Fig. 2. Sample taken after 75 min. of dialysis. Shadowed with chromium. Formation of FLS is further advanced. 'Spindles', thought to be tactoidal aggregates of tropocollagen particles, are particularly evident. Note fibril at lower left which appears to be forming from spindles which are aligned and possibly condensing upon the forming fibril. Magnification × 18,000.

Fig. 3. Sample taken after 60 min. of dialysis (different preparation than that from which Figs. 1 and 2 were made). Stained with phosphotungstic acid. Note single and multiple, thin long-spacing with dense spheroids at their ends, indicated by arrows. Magnification × 18,000.

PLATE 4

Stages in the formation of SLS by the action of ATP on a filtered 0·05 % acetic acid solution of ichthyocol.

Fig. 1. Sample taken immediately upon the addition of ATP (to make a concentration of 0·05 %) to ichthyocol filtrate. Shadowed with chromium. Note dense spheroids lying in a tangle of very thin filaments and 'spindles'.

Fig. 2. Sample taken immediately after addition of ATP (to make a concentration of 0·075 %) to ichthyocol filtrate. Stained with phosphotungstic acid. Note numerous dense spheroids and SLS (formed in a matter of seconds). Magnification × 18,000.

Fig. 3. Sample taken 30 sec. after addition of ATP (to a concentration of 0·2 %) to ichthyocol filtrate. Note SLS of varying thicknesses down to extremely thin ones, possibly composed of but a few tropocollagen particles. Magnification × 32,000.

COLLAGEN DISEASES

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In studying the programme of this Symposium it becomes obvious that my contribution has been included in order to provide some medical and biological background. I will therefore avoid exposing my ignorance of the fibrous proteins by reviewing in very broad terms some of the disease states in which connective tissue fibres become implicated.

In considering any connective tissue change it must be remembered that this tissue contains many cells in addition to the fibres and mucoid which make up the intercellular matrix. In embryonic connective tissues the cells are numerous and active and the intercellular matrix is scanty and poorly organized. In the adult tissues the cells are few, inactive and widely separated by the fibrous and mucoid matrix which endows the bone, tendon, cartilage or synovia with the physical properties that enable these structures to perform their supporting and lubricating functions. To perform these functions efficiently the matrix requires maximal physical stability but minimal metabolic activity. Indeed, metabolic activity is only required for the repair of damage caused by the operation of excessive mechanical forces, or for the remodelling of the supporting framework in response to alterations in the mechanical stresses it is called upon to resist.

Any irritant which produces an inflammatory reaction also provokes a reversion of the connective tissues to their embryonic state of cellularity, activity and scanty matrix. The extreme example of this is granulation tissue which is little more than a mass of cells and which is quite useless as a supporting structure or a lubricating fluid. This illustrates one of the important features of the supporting system, namely, that in the connective tissues increased activity and cellularity are often associated with decreased physical efficiency.

This reversion to an embryonic form is probably the most common connective tissue lesion and forms the basis of the connective tissue response during inflammation and repair resulting from mechanical and chemical injury or bacterial infection and a variety of other pathological processes. During recovery the supporting framework may be laid down again in its original form or it may be laid down in excess with resultant scarring, though sometimes the repair is inadequate, resulting in atrophy and loss of substance. Very little is known about the enzyme systems and other factors

that presumably control these fascinating processes, but they represent the most important disease of collagen.

The second and almost ubiquitous disease of collagen is represented by the ageing or degenerative process, in which there appears to be a gradual quantitative alteration in connective tissue components. With advancing age the mucoid and polysaccharide content of most connective tissues decreases, while the collagenous component increases and tends to be organized into a coarser fibre pattern. As a result these tissues become less hydrated, less elastic and resilient, and therefore less able to resist mechanical forces, and this is one reason why our limbs become stiff and readily damaged by trauma as old age creeps upon us. Cellular activity is notably absent in this process.

Then there are interesting diseases in which there is metaplasia of connective tissue, as, for example, in ankylosing spondylitis where the joint ligaments become ossified—turning the unfortunate patient into a living statue. Or the Ehler-Danlos syndrome where the collagen is replaced by elastin, creating the so-called india-rubber man. There are, of course, many other forms of metaplasia, which produce a variety of clinical syndromes, but very little is known about the factors which determine such changes, so that we are still largely unable to influence their course, although X-ray therapy does appear to have a favourable effect in ankylosing spondylitis.

Now I come to the collagen diseases in the sense in which this term is generally employed by the medical profession. These diseases have been loosely grouped together because they have certain features in common, such as severe constitutional illness, widespread inflammatory changes in the connective tissues and the production of fibrinoid. The fibrinoid change is found in minute scattered foci throughout the connective tissues in diseases such as rheumatic fever, rheumatoid arthritis, polyarteritis, lupus erythematosus and scleroderma. It can also be produced experimentally by the injection of heterologous protein and by mechanical trauma, and in arterioles it may follow a simple rise in blood pressure.

Fibrinoid areas can often be seen as opaque yellowish spots in the semi-translucent connective tissue. In histological preparations these areas are characterized by absence of connective tissue cells and blurring of the fibre pattern, which may be entirely replaced by amorphous material. The fibrinoid stains more intensely with eosin than the surrounding connective tissue and resembles slightly the appearance produced by deposits of fibrin, and some fibrin may in fact be present in the fibrinoid of rheumatic fever and other acute lesions.

In advanced rheumatoid lesions there appears to be no such admixture of fibrin, and in a structure like tendon a whole bundle of collagen fibres

may be replaced by amorphous material, which makes it clear that we are not merely dealing with the deposition of new material but a definite alteration of the connective tissue itself. At the edge of a fibrinoid area the thick collagen fibres can be seen passing into finer argyrophil reticulin fibres, and these in turn may disappear altogether in the amorphous lesions. Those parts of a fibrinoid area that contain silver-staining fibres also stain deeply with the periodic acid Schiff technique.

Such histologically defined fibrinoid was studied in Prof. Astbury's Department (1951), where it was shown that the amorphous fibrinoid tissue gave an X-ray diagram in which the collagen pattern was absent and replaced by a diffuse ring with a spacing of about 4·8 Å., and that samples from such a spot showed only amorphous material under the electron microscope. In the marginal areas of partial fibrinoid change there was excess of amorphous material together with broken and disintegrating collagen fibres.

The precise chemical characterization of fibrinoid has not yet been achieved, and, indeed, it is likely to be very complex, since the totally amorphous material is likely to differ from that containing silver-staining fibres; but it is generally agreed that a fibrinoid area contains much material that resists boiling, and that it has a low hydroxyproline content and a high tyrosine and hexosamine content. The chemical studies of Consden, Glynn & Stanier (1953) were, however, carried out on the fibrinoid of rheumatic fever, which differs in several respects from that found in rheumatoid disease. Furthermore, in disseminated lupus erythematosus there are also deposits of haematoxylin-staining material in the tissues which undoubtedly contain nucleoprotein, and Gueft & Laufer (1954) now suggest that the fibrinoid of this disease may be of a different nature and includes a protein residue of nuclear origin.

The fibrinoid change really represents a somewhat loosely defined histological appearance and probably includes a variety of connective tissue changes which may be produced by different mechanisms, so that there is unlikely to be a single simple answer to the problem of the collagen diseases. Furthermore, the fibrinoid change is by no means the most obvious feature of this group of diseases and may be no more than an interesting by-product.

Clinically the collagen diseases tend to be chronic or recurring maladies with marked constitutional illness and weight loss. Local lesions are found in the joints, blood vessels, heart, skin, muscle and the supporting reticulum of the internal organs. The clinical picture is largely determined by the distribution of these lesions and the tempo of the pathological process. The connective tissue lesions are essentially destructive so that

there is a tendency to atrophy and loss of substance in the supporting system. The spleen and lymph glands are often enlarged and there are marked changes in the plasma proteins with increase of the γ and α_2 globulin fractions and increase of mucoproteins and other substances which have not yet been fully characterized.

These features are common to all the collagen diseases, but there are material differences within the main group and some of these are summarized in Table 1.

Table 1

Disease	Etiology	Leucocytes	L.E. cells	D.A.T.	Therapeutic responses	
					Salicylates	Cortisone
Serum sickness	Heterologous serum	+E	−	−	o	++
Rheumatic fever	Group A streptococci	+	o	o	++	++
Periarteritis nodosa	?	+E	o	o	o	++
Rheumatoid arthritis	?	−	o	++	+	+
Disseminated lupus erythematosus	?	−−	++	++	o	++
Scleroderma	?	±	o	+	o	±

Serum sickness clearly results from the injection of heterologous serum in sensitized subjects; it tends to be a self-limiting process and does not recur unless further protein is injected.

Rheumatic fever follows infection with group A streptococci in susceptible subjects. Although the nature of this susceptibility is still unknown, rheumatic fever would disappear if streptococcal infection could be eliminated.

Periarteritis nodosa resembles serum sickness and rheumatic fever in many ways, and by analogy it should be caused by a similar hypersensitivity reaction, but unequivocal evidence of hypersensitivity has not been demonstrated in this disease, and very similar necrosis of the arterioles which affects the smooth muscle of the media as well as connective tissue is associated with a raised blood pressure in the absence of other disease.

These three conditions have much in common in that the pathological lesions are prominent in the arteries and small vessels though the heart valves and joint capsules are particularly affected in rheumatic fever. The inflammatory reaction is more or less acute with polymorphonuclear leucocytosis and a tendency to eosinophilia. All these processes appear to be completely or almost suppressed by cortisone in adequate dosage. Rheumatic fever is also almost completely suppressed by salicylates, where-

as these drugs have virtually no effect on periarteritis nodosa which is really a remarkable difference.

In rheumatoid disease, disseminated lupus erythematosus and scleroderma, no causal factors have been demonstrated, though there is just a suggestion of familial incidence, and in disseminated lupus erythematosus exposure to ultra-violet light will often flare up the disease process. All these diseases tend to be recurrent and progressive with widespread involvement of the supporting system. The inflammatory reaction is chronic or subacute and there is a tendency to leucopaenia which is most pronounced in disseminated lupus erythematosus; in lupus erythematosus there is a factor in the globulin fraction of serum which causes destruction of leucocytes producing the so-called lupus erythematosus cells, and this phenomenon is being widely adopted as a prime diagnostic criterion, but lupus erythematosus cells may be found in patients with severe arthritis or visceral lesions who have never had a skin rash, which leads to the delightful diagnosis of lupus erythematosus sine lupus erythematosus. The production of lupus erythematosus cells is presumably related to the occurrence of haematoxylin bodies in the tissues, and it has been suggested that the lupus erythematosus factor is an inhibitor of cellular desoxyribonuclease inhibitor (Kurnick, Pariser, Schwartz, Lee & Irwine, 1952).

Another interesting serum factor is that which produces agglutination of sensitized sheep cells. This factor, which appears to be present to some extent in the serum of normal individuals, is present in high concentration in the serum of most patients with rheumatoid arthritis and disseminated lupus erythematosus, and in some patients with scleroderma. Its nature has not yet been established, but it is mainly carried in the γ-globulin fraction.

The therapeutic responses in these diseases are also variable. Thus many features of rheumatoid arthritis are partially suppressed by both salicylates and cortisone, whereas disseminated lupus erythematosus is much more readily suppressed by cortisone while being largely unaffected by salicylates. Scleroderma, on the other hand, is virtually unaffected by any form of treatment.

This review has of necessity been very superficial, but it was intended to provide a rough panorama of the disorders of connective tissue for the chemists and physicists of this Symposium who are exploring the nature and behaviour of the fibrous proteins and to whom we medical men look for guidance in our attempt to understand many distressing diseases, with the ultimate aim of controlling and eliminating them altogether.

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THE CHEMISTRY OF KERATINOUS STRUCTURES

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Out of many possibilities, three aspects of the chemistry of keratinous structures have been chosen for consideration. These are problems of constitution, accessibility and reactivity, all of them being, of course, interrelated.

CONSTITUTION

At a meeting of this kind, the question which is uppermost in the mind of the chemist and technologist is whether the biologist can explain how and why Nature is able to maintain features of such surprising constancy in fibres, such as wool and hair, which display bewildering variations in composition.

Some 30 years ago, when research on wool was in its infancy, the chief emphasis was on variability of composition, particularly as regards sulphur-cystine content. Not only were different wools found to differ in sulphur content, but there were variations in sulphur content along the length of a single staple, between different types of cell, and between the cells and the intercellular phase. Then followed a period when variations in composition were ignored, attention being focused on the skeleton form of the molecular structure, which is qualitatively the same for all wools and capable of accommodating variations in composition. During the past few years, however, such variations have recaptured attention in two surprising ways.

The first concerns the plasticity of wool, which can be assessed very simply by following the rate of extension of single fibres under constant load (Ripa & Speakman, 1951). When, for example, a load of 700 kg./cm.^{2} was applied to a Lincoln wool fibre in water at 22·2° C., there was an immediate extension of 32%, which was followed by a slow creep until, after 10,680 min., the extension was 65·4%. In the later stages of extension there is a linear relationship between $\log(E-E_t)$ and t, where E is a limiting extension and E_t is the extension at time t (min.), and the slope k of this line gives a convenient measure of plasticity. When the undamaged root-ends of the fibres comprising a single staple of Lincoln wool were examined in this way, surprisingly large variations in plasticity were

observed, extreme values of k being $3{\cdot}83 \times 10^{-5}$ and $50{\cdot}2 \times 10^{-5}$. Similar results were obtained with other wools, mohair and human hair, and the cause of the variations was sought. It seemed possible that they were associated with the order of development of the follicles from which the fibres were derived, and the work of Dr F. W. Dry (1933) has made it possible to study the plasticity of fibres from different types of follicle. Using hogget's wool he found, for example, that fibres from primary central follicles have sickle-shaped tips, while those from primary lateral follicles have curly tips and those from the latest secondary follicles to develop, fibres known as histerotrichs, are only slightly wavy along the length. If account is also taken of the state of medullation of the tip and the length of the fibres, it is possible to classify fibres according to the type of follicle from which they are derived, and use was made of this procedure in obtaining the following values (Burley & Speakman, 1953) for the plasticity of fibres taken from a single lock of Lincoln hogget's wool. It will be seen that fibres from the first follicles to develop are the least plastic, and vice versa.

Table 1

Type of follicle	Fibre types	Plasticity (k) ($\times 10^{-5}$)
Primary central	Sickles	56·1
Primary lateral	Early curly-tips	68·6
Secondary	Late curly-tips	74·1
Secondary	Long histerotrichs	90·4
Secondary	Short histerotrichs	106

Similar results were obtained with other Lincoln wools, as well as with New Zealand Romney and Australian merino wools, and it is clear that there are gross differences between the fibres within a single staple of wool, differences which seem to be associated with differences in crystallinity and cross-linking.

Further dramatic emphasis on the inhomogeneity of wool comes from the work of Horio & Kondo (1953). Their discovery of bilateral asymmetry in wool fibres has been confirmed by Mercer (1953), who divides the fibre longitudinally into two approximately equal regions, one of which, the paracortex, is less vulnerable than the other, the orthocortex, probably because of a greater cystine content.

In striking contrast with such variability and heterogeneity, animal fibres display some features of surprising constancy. It has long been known that the combining capacity for acids, i.e. the content of basic amino-acids, is approximately constant, and Block has repeatedly emphasized that in the keratins the relative proportions of histidine, lysine and arginine are 1 : 4 : 12. Further, Middlebrook (1951) and Tibbs (1951), using Sanger's technique

(1945), have shown that the terminal amino groups of the polypeptide chains of fibres as different as merino wool and human hair are provided by the same seven amino-acids, viz. glycine, alanine, valine, serine, threonine, aspartic acid and glutamic acid. Not only so, but the molecular weight of the chains, as deduced from the amount of terminal amino-nitrogen, is always approximately the same on the average, as shown in Table 2.

Table 2. *Terminal amino-acids in four keratin fibres*

(Amounts in millimoles per kilogram)

Amino-acid	Lincoln wool	New Zealand Romney wool	Australian merino wool	Human hair
Glycine	5·2	4·5	4·7	3·9
Alanine	1·3	1·2	1·2	1·0
Valine	2·4	2·4	2·4	4·0
Serine	1·3	1·2	1·2	1·0
Threonine	4·8	4·9	4·9	4·0
Aspartic acid	0·6	0·6	0·6	0·5
Glutamic acid	1·3	1·2	1·2	1·0
Total	16·9	16·0	16·2	15·4
Average chain weight	59,000	62,500	62,000	65,000

As stated at the outset of this section, the question which the chemist wishes to ask the biologist is whether he can throw light on the mechanism and reason for maintaining such constancy of composition, particularly as regards basic and terminal amino-acids, in structures which are otherwise characterized by such extreme inhomogeneity and variability.

ACCESSIBILITY

The reactivity of the protein components of keratinous fibres depends not only on the relative proportions of the constituent amino-acids and the position of the amino-acid residues relative to one another in the polypeptide chains, but also on the accessibility of peptide groups and reactive side-chains to reagents of varying molecular size in different media. In general, it may be stated that accessibility will be determined by the configuration of the chains and their arrangement with respect to one another, the relative proportions of ordered (crystalline) and disordered (amorphous) material being particularly important.

The accessibility of a keratinous fibre to any reagent is, of course, increased when the structure is swollen, and the most important swelling agents are water, methanol, ethylene glycol, formic acid, phenol and concentrated aqueous solutions of hydrogen bond breakers, such as urea and lithium bromide. As was indicated many years ago, it is here that the chief

difficulty in modifying keratin by chemical means is encountered, for the reagents which are most likely to be reactive with keratin are themselves attacked by the solvent from which it would be most desirable to apply them. For this reason it became important early in the study of the reactivity of keratin to have some measure of the maximum size of molecule which would be capable of penetrating the *fine* structure of the dry, unswollen fibre.

Table 3

Medium	Work for 30 % extension (g.cm./c.c.) × 10^5
Water	1·43
Methyl alcohol	1·72
Ethyl alcohol	2·44
n-Propyl alcohol	4·43
n-Butyl alcohol	5·01
n-Amyl alcohol	5·02
Dry air	5·37

Table 4

Medium	Time of immersion (days)	Work for 30 % extension (g.cm./c.c.) × 10^5
Water	1	1·62
n-Propyl alcohol	1	4·38
n-Propyl alcohol	14	4·42

In a first attempt to determine the accessibility of dry wool fibres, their resistance to extension in a series of dry, primary alcohols was determined at 22·2° C. (Speakman, 1930). The fibres were stretched after 1 day's immersion in the alcohol and the results are given in Table 3. Since the work required for 30% extension in *n*-butyl and *n*-amyl alcohols is almost the same as in dry air, and since *n*-propyl alcohol has only a slight weakening effect on the fibres, it was concluded that the fine structure of dry wool fibres is inaccessible to molecules larger than those of *n*-propyl alcohol at 22·2° C. Doubt as to the validity of this conclusion has been aroused recently by King's (1947) discovery that the rate of adsorption of *n*-propyl alcohol from a saturated atmosphere at 25° C. is small, and as much as 16% is adsorbed in 8 days. It has, therefore, been supposed (Alexander, Carter, Earland & Ford, 1951) that no penetration would be detected in the 1 day allowed in the experiments where the fibres were immersed in the alcohol. This supposition is disproved by the data of Table 4 (Jagger & Speakman, 1952), which show that the resistance of human hair fibres to extension in *n*-propyl alcohol is the same after 14 days' as after 1 day's immersion.

It is, however, surprising that as much as 16% of *n*-propyl alcohol can be adsorbed by wool without marked diminution in its resistance to extension, and it seemed desirable that confirmation of the diminishing accessibility of the fibre to alcohols of increasing molecular weight should be obtained by an independent method of experiment. The adsorption isotherms of wool and water, methyl alcohol, ethyl alcohol and *n*-propyl alcohol were, therefore, determined at 25° C. (Nicholls & Speakman, 1954). It is noteworthy that whereas equilibrium was reached in 2–4 days with methyl alcohol, as much as 3 months was needed at higher partial pressures and 6 months at lower partial pressures with *n*-propyl alcohol. The adsorption isotherms are reproduced in Fig. 1.

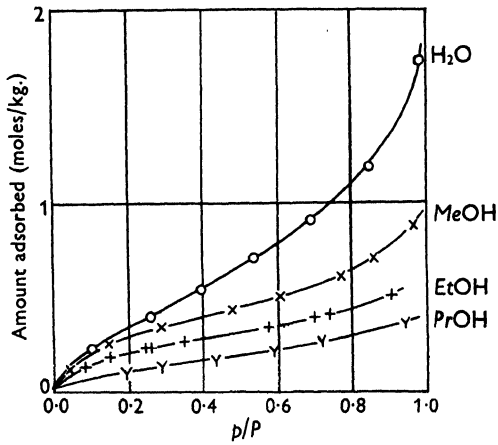

Fig. 1.

On analysing the isotherms by means of the Hailwood-Horrobin (1946) equation,

$$\frac{Mr}{100m} = \frac{\alpha h}{1-\alpha h} + \frac{\alpha\beta h}{1+\alpha\beta h},$$

where r is the percentage by weight of water or alcohol sorbed, m is the molecular weight of the adsorbate, h is the partial pressure of the vapour expressed as a percentage, α and β are constants, and M is the molecular weight of the unit which combines with one molecule of the adsorbate, it was found that the value of M increases with increasing molecular weight of the adsorbate. In all cases, the value of M was greater than the average residue weight R, and according to Hailwood & Horrobin a value for the fraction of the fibre which is accessible to the adsorbate is given by R/M. The results are given in Table 5, R being taken as 122. The fraction of the wool which is accessible to the adsorbate decreases with increasing size of the exploratory molecule, but the amount of *n*-propyl alcohol adsorbed

from the saturated vapour is far greater than would be expected from the change in elastic properties. Since the ordered and disordered regions of wool are in series and in parallel with one another, the two sets of results might be reconciled if most of the alcohol is adsorbed by the disordered regions or held in capillary spaces, leaving the elastic properties of the crystalline regions unaltered.

Table 5

Adsorbate	M	Fraction accessible (R/M)
Water	175	0·70
Methyl alcohol	307	0·40
Ethyl alcohol	380	0·32
n-Propyl alcohol	430	0·28

Table 6

Adsorbent	M	Fraction accessible (R/M)
Lincoln wool	175	0·70
Romney wool	191	0·64
Merino wool	194	0·63
Mohair	203	0·60

It will be evident from the preceding observations that a complete understanding of the properties of wool fibres is impossible without a knowledge of the proportions of amorphous and crystalline material present. If a molecule as small as that of *n*-propyl alcohol is able to discriminate between amorphous and crystalline regions, even greater discrimination will be shown by larger molecules, such as those of acid dyes, if the differences of accessibility persist in aqueous media. Two attempts have, therefore, been made to determine the relative proportions of amorphous and crystalline material in wool in water.

The first is a simple repetition of Hailwood & Horrobin's analysis of the water-adsorption isotherms of three wools and mohair at 25° C., and the results are given in Table 6 (Nicholls & Speakman, 1954). Doubt as to whether the absolute values for the fraction accessible could be regarded as correct was aroused when the water-adsorption isotherm for Lincoln wool which had been esterified with methyl alcohol was determined and analysed (Nicholls & Speakman, 1954). In this case, the value of M was 194, compared with the 175 for untreated wool, and the fraction accessible was only 0·59, compared with 0·70 for untreated wool. The presence of more bulky side-chains in the esterified wool, and any main chain hydrolysis

that may have occurred during esterification, should have had the effect of decreasing crystallinity (increasing accessibility). It seems probable that the result for esterified wool is misleading because methylation of the free carboxyl groups reduces their affinity for water and, therefore, the amount of water adsorbed per average residue weight, thus giving the same effect as a decrease of accessibility. If this is the case, the preceding analysis of the water-adsorption isotherms of untreated fibres may indicate their relative degrees of crystallinity, but is unlikely to give correct quantitative values for the fractions accessible to water molecules. An independent method of determining the amorphous/crystalline ratios of the same untreated fibres was, therefore, evolved (Speakman, 1953).

Table 7

Fibre	Gain in dry weight (%)	Fraction accessible
Lincoln wool	1·41	$0{\cdot}87_3$
Romney wool	1·36	$0{\cdot}84_2$
Merino wool	1·35	$0{\cdot}83_6$
Mohair	1·33	$0{\cdot}82_4$

The general principle of the method is to determine the gain in dry weight of wool after exposure to saturated deuterium oxide vapour. If the whole of the fibre were freely accessible to deuterium oxide the theoretical gain in weight, calculated from the amino-acid analysis, which is now reasonably complete, would be 1·615% when the hydrogen-deuterium exchange is complete. Samples of the purified fibres were suspended from a quartz spiral balance, and after their dry weights had been determined over phosphorus pentoxide *in vacuo*, the wools were exposed to saturated deuterium oxide vapour and then re-dried. The process was repeated several times until no further change in dry weight was detected, and the results obtained are given in Table 7, which also includes values for the fraction accessible to deuterium oxide, obtained by expressing the observed gain in weight as a fraction of the theoretical. These values (Nicholls & Speakman, 1954) are more reliable than those obtained in earlier, preliminary, experiments (Speakman, 1952, 1953), because a more sensitive apparatus was used with larger samples of wool. Although the order of accessibility given by the hydrogen-deuterium exchange reaction is the same as that found by analysing the water-adsorption isotherms, the exchange reaction suggests that the different fibres do not vary greatly in accessibility and gives very much higher values for the fraction accessible to water. Because of its simplicity, the direct gravimetric method of deter-

mining accessibility seems more reliable, but it must be remembered that the results of Table 7 are based on two assumptions. The first, that the theoretical gain in weight is the same for all the fibres examined, is not likely to be seriously in error, but the second, which is a tacit assumption that the amorphous and crystalline regions have the same composition, gives more anxiety. In the case of silk, for example, it is known that the amino-acids with the more bulky side-chains preponderate in the amorphous phase, and the same may be expected with keratin. It does, however, seem likely that keratin is as accessible to water as the data of Table 7 indicate, because, whereas the tyrosine side-chains of wool can be almost fully iodinated (96%) with an alcoholic solution of iodine at 25° C. (Richards & Speakman, 1953), reaction with the tyrosine side-chains of silk, which is far more crystalline than wool, is far from complete under similar conditions. In other words, even if all the tyrosine in silk is present in the amorphous phase, its accessibility is impaired by neighbouring crystalline regions, and a similar result would be expected with keratin if its crystallinity were as high as the data of Table 6 suggest. The degree of accessibility indicated by the hydrogen-deuterium exchange reaction is, therefore, regarded as being approximately correct, but final values, based on the reaction, must await the results of amino-acid analyses of the crystalline and amorphous phases of keratin.

If, as the data of Table 7 indicate, the fraction of wool which is inaccessible to water at room temperature is as small as 13–16%, the fraction which is inaccessible in boiling, dilute solutions of sulphuric acid (pH 3) must be even less, and one of the more baffling features of the dyeing of wool with acid dyes can now be explained. During the past 40 years a large body of evidence has been accumulated to show that the combining capacity of wool for acid dyes is approximately the same as for simple acids, such as hydrochloric acid, suggesting that the coloured ions have no serious difficulty in gaining access to the basic side-chains. In sharp conflict with the chemical evidence, however, Astbury and Dawson (1938) found that wool dyed to saturation with Crystal Ponceau gave a normal α-photograph, indicating that the dye had not penetrated the crystalline phase of the fibres; and, unlike simple organic acids, dye-acids were incapable of delaying or preventing setting of the crystalline phase of stretched fibres in steam, as would be expected if the phase were accessible to dye-acids. Although the dye-acid of Coomassie Milling Scarlet G (Colour Index, no. 443) was subsequently found to be capable of delaying the setting of the crystalline phase (MacArthur, 1946), the main difficulty remained: that despite the large amounts of dye combined with the fibres, their photographs revealed no trace of dye crystallites, nor was there any significant distortion of the

normal photograph of unstretched fibres. In fact, wool dyed with Coomassie Milling Scarlet G gave an α-photograph of such excellent definition as to suggest that combination with dye had an orienting effect on the structure. It now seems clear that the X-ray observations on dyed fibres relate to only a very small fraction of the structure, and as the combining capacity of wool for dye-acids is only approximately constant, there seems now to be no conflict between the chemical and physical evidence regarding the mechanism of acid dyeing, especially if the basic side-chains occur preferentially in the amorphous phase.

REACTIVITY

Since the proportion of amorphous material in keratinous fibres is so great, there are correspondingly great opportunities for modifying their properties by cross-linking and other reactions. At the present time the chief obstacle to progress in this field is lack of knowledge of the arrangement of the amino-acids in the polypeptide chains. Such knowledge is important for two main reasons: first, because the reactivity of the peptide bonds, side-chains and cross-linkages is profoundly affected by their environment; and, secondly, because the truly constructive study of cross-linking is impossible if the relative positions of the side-chains which are to be bridged are unknown.

Some indication of the importance of environment on reactivity is afforded by the fact that peptide bonds involving the amino group of serine are known to be particularly labile (Casal & Desnuelle, 1948), and the controlled hydrolysis of wool keratin has shown that particular amino-acids are liberated preferentially, their nature and proportions depending on the pH value of the solution used (Biserte & Pigache, 1951, 1952). In addition, Lindley & Phillips (1945) have argued that the fraction of cystine which is reactive with sodium bisulphite is associated with a polar environment. Important new light on this suggestion comes from recent work at Oxford on the action of sulphites on cystine and other disulphides (McPhee, private communication). It appears that it is the sulphite ion which is reactive at all pH values, the extent of reaction being reduced by the presence of $-COO^-$ ions in the neighbourhood of the disulphide bond. Now it is known from the work of Consden, Gordon & Martin (1949) on the partial hydrolysates of wool that part of the cystine is combined with dicarboxylic amino-acids, and it seems clear that the Oxford work provides a satisfactory explanation of the influence of pH on the reaction between wool and sulphite solutions in terms of the concentration of sulphite ions and the state of ionization of carboxyl groups in the neighbourhood of the disulphide bonds.

From these observations on the influence of environment on reactivity it is possible to suggest a second possible explanation of a curious discrepancy between the results of two earlier studies of the action of sulphites on wool. Phillips (1938) has shown that the maximum combination of sulphite and wool occurs at pH 5, whereas earlier work (Speakman, 1936) on the setting of stretched fibres in sulphite solutions had shown that the maximum effect is obtained at pH 6. The difference in pH value has been attributed to the fact that the setting experiments were carried out with fibres which had been stretched 40%, and that at pH 6, where salt linkages are most stable, the disulphide cross-linkages are most strained and, therefore, most reactive. A second possibility is that the environment of the disulphide bonds may be altered during extension of the fibres, and if the acidic side-chains are removed by rotation or translation, or if basic side-chains are brought nearer by the same means, reactivity would be enhanced and the pH at which reaction would be most pronounced would be displaced to a higher value than for unstretched fibres.

Turning now to cross-linking, all work has so far proceeded on an empirical basis because so little is known about the relative positions of reactive side-chains in keratin. The most successful cross-linking agents are those which react with basic and amide side-chains, which are present in such abundance. Of these reagents, the simplest is formaldehyde, which, according to Fraenkel-Conrat & Olcott (1948), can form methylene bridges between amino groups on the one hand and primary amide or guanidyl groups on the other. This conclusion was drawn from experiments with simple compounds, but in experiments with proteins at pH 3–8 at room temperature it was shown also that methylene bridges are formed between amino and other reactive groups, the important part played by the amino groups being shown by the fact that proteins which are rich in amide but poor in amino groups are not readily cross-linked under the above conditions. Under acid conditions, however, Haworth, Peacock, Smith & MacGillivray (1952) have found that cross-linking is probably due to the formation of methylene bridges between amide groups and aromatic nuclei (tyrosine). Alexander, Carter & Johnson (1951), on the other hand, have suggested that methylene bridges are formed between tyrosine and lysine side-chains when wool is treated with formaldehyde for 30 min. at 60° C. and pH 6–7. There is no necessary conflict between this conclusion and that of Haworth *et al.*, because the pH and temperature of treatment were different in the two cases, but recent work indicates that Alexander *et al.* may be mistaken. As has already been indicated, the tyrosine side-chains of wool can be almost fully converted (96·3%) into 3 : 5-diiodotyrosine side-chains by treatment with an alcoholic solution of iodine for 72 hr. at

22·2° C. If, when wool is treated with formaldehyde under Alexander's conditions, methylene bridges are formed between lysine and tyrosine side-chains, the treated wool should show a diminished combining capacity for iodine. The amounts of iodine which combined with untreated and formaldehyde-treated wool were, however, 64·5 and 63·2 mg. per g. of wool, corresponding to the conversion of 96·3 and 94·4% of the tyrosine into 3 : 5-diiodotyrosine. It seems unlikely, therefore, that cross-linkages are formed between tyrosine and lysine under the conditions used.

Benzoquinone is much more effective as a cross-linking agent than formaldehyde, but even more striking results have been obtained recently with certain triketones (Cockburn, Jagger & Speakman, 1953). The first of these to be examined was ninhydrin, and its effectiveness is illustrated by the following data: human hair fibres, after being boiled for 1 hr. in a 1% (0·056 M) solution of ninhydrin, showed an increase in resistance to 25% extension in water at 22·2° C. of 26·2%. With *peri*-naphthindantrione under similar conditions the increase in resistance to extension was 38·9%, the greatest strengthening so far obtained by cross-linking reactions. Reagents of this kind are effective also with protein synthetic fibres. When, for example, Ardil fibres are boiled for 1 hr. in a 1% solution of ninhydrin (pH 4·04), the resistance to 25% extension in water at 22·2° C. is 1409 kg. cm.$^{-2}$, compared with 674 kg. cm.$^{-2}$ for untreated fibres and 534 kg. cm.$^{-2}$ for fibres which had been boiled in a buffer solution at pH 4·09 for 1 hr. before extension in water. Since ninhydrin has no strengthening action on fully deaminated fibres, it seems clear that the basic side-chains are involved in the cross-linking reaction, but its exact nature is not yet known. It is, however, interesting that negative results are obtained with 1 : 2-diketohydrindene, 1 : 3-diketohydrindene, triquinoyl and alloxan.

A second method of reinforcing animal fibres is to convert the disulphide bonds into cross-linkages of greater stability. Unfortunately, it is not possible to use potassium cyanide for this purpose because, although the disulphide bonds are converted into lanthionine cross-linkages, the fibres are weakened by main chain hydrolysis. The classical method of modifying the disulphide bonds consists in interposing either a metal atom or an organic grouping between the two sulphur atoms (Speakman, 1934). For this purpose, the fibres are first treated with a reducing agent to form cysteine side-chains and then with either a metal salt or an organic polyhalide. Methods of this kind were first proposed in 1934, by Speakman, and the second, involving the use of organic polyhalides, was afterwards the subject of detailed investigation by Harris (1942). More recently, he has shown that the damage suffered by wool during treatment with sodium hydrosulphite is minimized when ethylene dibromide is present (Harris &

Rainard, 1950), presumably because the cysteine side-chains are reunited by reaction with the dihalide. Another series of reactions, in which disulphide bond breakdown and linkage rebuilding are brought about in a single treatment, is now available. In this case, however, disulphide linkages are re-formed from cysteine side-chains and enhanced stability is not obtained. Nevertheless, the reactions are not without interest, and they should perhaps find a place in this section because they afford another interesting example of action of triketones on keratin. If, for example, fibres at 30% extension are treated with a solution of ninhydrin (1%) in sodium sulphite (0·5 M) for 1 hr. at 50° C., they acquire a set permanent to release in boiling water. Since ninhydrin is capable of oxidizing thiophenol to diphenyl disulphide, it seems clear that the set is obtained by two successive reactions: disulphide bond breakdown by the sulphite solution at pH 10, which causes dissipation of stress, followed by oxidation of the cysteine side-chains to disulphide cross-linkages by the ninhydrin. Similar results have been obtained with other triketones, including *peri*-naphthindantrione and, surprisingly enough in view of their behaviour with untreated fibres, triquinoyl and alloxan.

The third method of reinforcing the amorphous phase of animal fibres is by the formation of internal deposits of polymer. Among the earlier methods used for this purpose was one in which the fibres were first impregnated with a dilute solution of ferrous ammonium sulphate and then treated with a mixture of hydrogen peroxide and monomer, preferably at low pH values in absence of oxygen (Lipson & Speakman, 1949). A two-stage process of this kind has obvious disadvantages, and in seeking a simplified procedure it was found that impregnation with ferrous ammonium sulphate is unnecessary if hydrogen peroxide is replaced by potassium persulphate (Madaras & Speakman, 1954). Polymerization is initiated by the free radicals which are formed by the action of potassium persulphate on the cystine disulphide bonds:

$$R\text{—S—S—}R \rightleftarrows 2\ R\text{—S}\cdot,$$

$$R\text{—S}\cdot + S_2O_8^{2-} \rightarrow R\text{—S—O—}SO_3^- + \cdot SO_4^-,$$

$$R\text{—S—O—}SO_3^- \rightarrow R\text{—S}\cdot + \cdot SO_4^-,$$

$$\cdot SO_4^- + H_2O \rightarrow HSO_4^- + \cdot OH.$$

When a 1·5 g. sample of wool was treated with a mixture of 0·2 N-sulphuric acid (50 c.c.), methacrylic acid (2·5 c.c.) and 0·027 g. potassium persulphate for 1 hr. at 25° C. in an atmosphere of nitrogen, the gain in weight due to internal deposition of polymer was 49·4%. Under similar conditions, but using water in place of sulphuric acid solution, the gain in weight was 35·1%.

Perhaps the most interesting observation on the reactivity of the disulphide bond of wool concerns the behaviour of stringently de-aerated solutions of monomer in reaction with the fibre (Crawshaw & Speakman, 1954). When wool (0·05 g.) was treated with 10 c.c. of a 5% (v/v) solution of methacrylic acid in 0·1041 N-sulphuric acid for 14 hr. at 25° C. in absence of light, the contents of the flask remained fluid, but exposure to diffuse light for a further 6 hr. caused the formation of a gel of polymer. In presence of diffuse light, polymerization of methacrylic acid can likewise be promoted by cystine and ethyl disulphide, but not by cysteine, lysine or iodogorgoic acid, and it was concluded that the free radicals which initiate polymerization are formed by homolytic fission of the disulphide bonds of both wool and simple disulphides:

$$R\text{—S—S—}R \overset{h\nu}{\rightleftarrows} 2\,R\text{—S}\cdot.$$

Assuming this to be the case, simple new methods of stabilizing the disulphide bonds of wool at once suggest themselves, and promising results have already been obtained in preliminary experiments in this field.

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THE PROTEINS OF THE MYOFIBRIL

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As examples of the fibrous group of proteins, keratin and collagen are perhaps the most typical if only for the reason that they are visibly fibrous and endowed with the mechanical properties which we associate with a true fibre. There are many soluble proteins, however, which can be considered fibrous because in the first place they are essentially asymmetric, and, in addition, under defined conditions, they can aggregate into macroscopic forms which are fibrous both visibly and mechanically. These proteins are important because unlike the insoluble fibres they can be studied after rigorous purification. Some of them, moreover, play fundamental roles in the animal organism—fibrinogen, the agent of blood-clotting, and the fibrous muscle proteins which underlie the very act of contraction. These latter will be considered in the present contribution, first as a prelude to the cytological and functional aspects discussed by Dr Perry and Prof. Weber respectively, and second for the significance they may have in explaining some puzzling features of cellular structure and organization.

In muscle, four different systems can be distinguished whose properties, function and amount vary considerably with the type of muscle: the proteins of the contractile material itself, the proteins of the sarcoplasm, the proteins associated with discrete granules, and the insoluble extracellular proteins—reticulin which encases the individual fibres, and collagen which connects the fibres ultimately with the muscle insertions. These systems are discrete even in the physical sense, for the contractile units do not seem to contain many of the glycolytic enzymes associated with the sarcoplasm, and the latter, in turn, is lacking in the respiratory enzymes which are particularly associated with the muscle granules. Except in passing mention, none of these systems will be discussed except the myofibril, and most of the discussion will centre on mammalian skeletal muscle about which much is known.

PROTEINS OF THE MYOFIBRIL AND THEIR EXTRACTION

In skeletal muscle, the muscle mass can be comminuted to morphological units no wider than 1 μ which are virtually free of glycolytic enzymes and which possess much of the histological detail of the original fibre. These

units are called the myofibrils, and the threads of which they are composed, the filaments. Considering only the muscle fibre itself and not the stroma or the extracellular space, there are in 100 ml. of fibre volume about 11 g. of myofibrillar protein (see Bailey, 1948*a*), and accepting a division into sarcoplasmic and fibrillar space, the absolute concentration of protein in the myofibril must be at least 20 g./100 ml.—assuming a uniform distribution within the myofibril itself, which is certainly not the case. In the anisotropic band, which is now known to contain far more protein than the isotropic, the concentration of protein must be even higher. The properties of gels of this concentration are really not known, though we often ascribe to muscle properties which pertain only to dilute systems. Portzehl & Weber (1950) have shown indeed that the behaviour of 'model' actomyosin threads change fundamentally when the protein concentration is increased from 2 to 10%, and no doubt some of the peculiarities observed in the extraction of fibrillar protein might be explained by the physical nature of the gel. Electron-optical and X-ray studies point to a great regularity in the structure of the myofibril, extending down to the molecular level. This is understandable not only from the ability of asymmetric molecules to align themselves, but also from the limited water content which is comparable to that of classical crystals such as edestin and lactoglobulin.

In recent years various methods have been devised for the isolation of the myofibrils in the physiologically active state (Schick & Hass, 1949, 1950; Perry, 1951), and these have been shown to consist predominantly of actin, myosin and tropomyosin (Perry, 1953). A fourth component which was not characterized, but which could have been the 'inactive' form of actin (see below), was found to leach out slowly in dilute buffer. There is no certain evidence, however, that only three proteins are contained in the isolated myofibril, though they must occupy most of it. The accretion of material which composes the *Z* band, for example, has never been characterized, though it is almost certain to consist of protein.

The methods for extracting the myofibril proteins have become increasingly complex. Originally, the general principle was to extract fresh, coarsely minced muscle with neutral or slightly alkaline salt solution, and to dilute the extract below the salting-in threshold of the 'myosin' which precipitated, leaving the sarcoplasmic proteins in solution. The researches of the Szeged school under Szent-Györgyi and Straub have shown that such preparations of myosin contained a hitherto undescribed protein, actin, and that the precipitate was in reality a complex of actin and myosin, called actomyosin (see Szent-Györgyi, 1951). By studying the exact details of preparation made by various authors in the pre-actin era, it is clear that the actin content varied greatly, some preparations containing very little indeed

(see Bailey, 1954). Reasonably pure myosin can be obtained by short extraction of the muscle mince at pH 6·5, followed by several re-precipitations (Banga & Szent-Györgyi, 1941–2). Longer extractions give actin-containing preparations, particularly at a more alkaline pH.

The original preparations of actin (Straub, 1942, 1943) and of tropomyosin (Bailey, 1948*b*), devised independently, seem to have made use of the same principle, viz. the initial treatment of the muscle debris with organic solvents to denature the myosin component and subsequent aqueous extraction of the dried residue. Tropomyosin differs from actin, however, in that its interaction with some component or components of the debris is strong enough to prevent extraction with water, and strong salt solutions need to be used; actin, on the other hand, is extracted in the entire absence of salt.

Hasselbach & Schneider (1951) have brought to light some interesting features concerning the differential extraction of the muscle proteins, which stress the importance of the degree of comminution, a fact long ago emphasized by Bate-Smith (1934). If inorganic pyrophosphate is added to a salt solution suitable for the extraction of myosin, it helps to dissociate the actin from the myosin which now passes quantitatively into solution. Evidence is accumulating that the bulk of the myosin resides in the *A* band and can pass out through the damaged sarcolemma and reticular network which surround the fibres more easily than can the actin, which appears to ist in the form of long threads stretching through the *I* band to the band (Hanson & Huxley, 1953). When now the residue, after losing its myosin, is finely homogenized, the actin (and presumably the tropomyosin) can also be extracted without changing the composition of the extracting solution. According to Weber & Portzehl (1952), it is simply the physical effects of comminution, both on the actin thread and on the reticulin sheath which allow the extraction. Tsao & Bailey (1953) have elaborated this method to give myosin, actin and tropomyosin from a single sample of muscle mince.

Several workers, including Hasselbach & Schneider (1951), have attempted to assess the amounts of the various protein components of skeletal muscle. Any worth-while discussion of the results should be preceded by a detailed consideration of the methods used (Weber & Portzehl, 1952; see Bailey, 1954), but it does seem that the probable content of actomyosin in adult rabbit or chick muscle is 60% of the total *intracellular* protein; tropomyosin comprises some 5% and the sarcoplasmic proteins 35%.

Herrmann & Nicholas (1948) and later Robinson (1952*a*, *b*) have attempted to follow the pattern of development in embryonic muscle. In the 14-day chick embryo, the myofibrils are characterized by large, centrally placed nuclei and somewhat primitive striations. The contribution of

nuclear nucleoprotein at this stage is very great indeed—some 65% of the total nucleic acid plus protein (Robinson, 1952*b*). By contrast, the truly contractile material comprises only 7% of the total, yet suffices to endow the fibre with contractility if elicited by direct stimulation. In adult muscle, as we have seen, the contribution of the actomyosin is 60% of the total intracellular protein, whilst the nucleoprotein contribution is virtually negligible—of the order of 1%. Fig. 1 summarizes Robinson's data for the chick, from the 13th day of incubation to the adult stage.

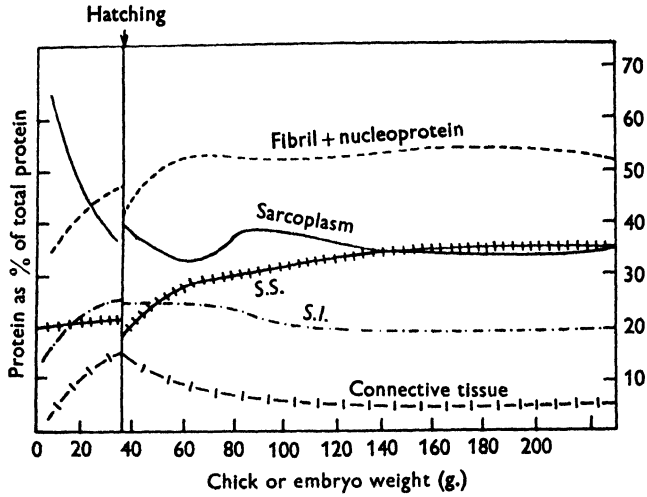

Fig. 1. Changes in chick-muscle proteins during development (adapted from Robinson, 1952*a*, *b*). Continuous line represents the water-soluble sarcoplasmic material. *S.S.* = salt-soluble but water-insoluble fraction (actomyosin, nucleoprotein); *S.I.* is the salt-insoluble intracellular fraction (denatured actomyosin?). These two curves are added together to give top curve (– – –) which at the adult stage represents the contractile material.

PHYSICAL PROPERTIES OF MYOSIN, TROPOMYOSIN AND ACTIN

Apart from the fact that all three proteins can aggregate into polymeric fibrous forms, their classification as fibrous could also be justified by the asymmetry of the fundamental units themselves. Such units must be thought of as the simplest form of the protein which is functional. For example, Tsao's experiments have defined three forms of actin which we can call *A*, *B* and *C*, of which *B* is a dimeric form of *A* and *C* a thixotropic form of *B*. *C* is thought to be the functional form of actin in muscle itself. Now *A* may itself consist of subunits, held together by loose secondary valence forces, or by strong —S—S— bonds as in the case of insulin; this is not yet known. It is known, however, that treatments likely to liberate

the subunits themselves would destroy the ability of actin to give form *C*. In such a case, form *A* would be considered the fundamental native protein molecule, or the fundamental kinetic unit. The term 'subunit' carries with it the connotation that it is the ultimate unit which can be derived from a protein without the rupture of *peptide* bonds; but there may be stages in the aggregation of the subunits which give more complex products before the functional form of the protein is elaborated. For proteins in which this latter tends itself to polymerize, it is often convenient to borrow from polymer chemistry, and to employ the terms monomer, dimer and so on.

Myosin. Myosin, with the highest particle weight of the three (850,000), is thought by Portzehl (1950) to be 2300 Å. long by only 23 Å. wide. This estimate, made from the frictional ratio on the assumption that the particle is *rigid*, is difficult to accept. It is easier to believe that a particle of such tenuity would take up coiled or helical configurations which vary with the solution parameters. *In situ*, of course, the marked anisotropy of the A band does suggest that it exists in oriented form, as does the α-type X-ray diffraction pattern (Astbury, 1947).

Tropomyosin. Tropomyosin has been studied by several physical methods, viscosity, sedimentation-diffusion, light-scattering (Bailey, Gutfreund & Ogston, 1948; Tsao, Bailey & Adair, 1951; Doty & Sanders, private communication). The monomer of particle weight 53,000 is thought to be about 400 Å. long and 15 Å. wide. This width is no more than might be expected from two α-type chains lying side by side, and since end-group assay by Sanger's fluorodinitrobenzene (FDNB) method has revealed no *N*-terminal acid (Bailey, 1951), the molecule was formulated as a closed loop. The carboxypeptidase method has since shown beyond all doubt that a *C*-terminal end is present (isoleucine), and by following the sequential liberation of amino-acids, Locker (1954) has derived a sequence of six amino-acids from the *C*-terminal end. Actin likewise possesses a *C*-terminal acid (phenylalanine) but no *N*-terminal (Locker, 1954). Results such as these are difficult to interpret and are a very real challenge to those interested in chemical aspects of protein structure. They may signify that FDNB is inaccessible to some α-amino groups, or it may be that some DNP-amino-acids in peptide linkage suffer extensive destruction during hydrolysis. Neither of these explanations seems probable, however, in the case of the interesting B peptide liberated from fibrinogen during clotting (Bettelheim & Bailey, 1952), for steric hindrance seem less likely in a polypeptide, and even with the shortest hydrolysis times, no trace of *N*-terminal acid can be detected. The possibility still remains, therefore, that tropomyosin is a closed loop with a branching point at an α or ω carboxyl, according as the loop is closed by a corresponding ω or α carboxyl (Bailey, 1953).

One of the most striking properties of tropomyosin is its ability to form linear aggregates when salt ions are removed, an effect which causes a very large rise in viscosity (Bailey, 1948*b*). It was first thought from the results of electron microscopy (Astbury, Reed & Spark, 1948) that such an end-to-end aggregation was accompanied also by side-to-side, since the fibrils obtained were of considerable size (3000 × 250 Å.). Light-scattering studies, however, have shown that up to the formation of a decapolymer at least the aggregation is linear (Doty & Sanders, private communication), and the electron-visible aggregates must be produced by drying. The polymerization is probably electrostatic in origin, since it is profoundly influenced by salt ions and could be adequately explained if the tropomyosin rod embodies a local concentration of positive charges at one end and negative at the other. The phenomenon may be relevant to the role of tropomyosin in muscle, which is yet unknown, but it is interesting as a basis for the *spontaneous* biogenesis of fibrous molecules from smaller units. Actin behaves in the opposite manner; the so-called fibrous form, whatever its exact nature (see below), is produced only in presence of salt ions, particularly those of divalent metals.

Its unusual properties have made tropomyosin more interesting to the protein chemist than to the muscle biochemist. Whilst in absence of salt it polymerizes, in presence of salt at ionic strength 0·4 it can readily be crystallized by slight acidification to pH 6 (Bailey, 1948*b*). These crystals contain about 90% of water and are extremely fragile. Hamoir (1951) has devised a method of extraction from fish muscle which brings out a complex of tropomyosin and nucleic acid, and Tsao (unpublished) has obtained such complexes from skeletal, uterine and cardiac muscle. Nucleotropomyosin crystals are more robust and less soluble than pure tropomyosin, and some of Tsao's crystals have grown to a length of 5 mm. It seems unlikely from the known nucleic acid content of the myofibril (Perry, 1953) that tropomyosin exists *in situ* as a complex; it is more probable that the complex is formed during extraction.

If all the salient properties of tropomyosin are considered together—that it is both fibrous and classically crystalline, that in the native state it gives a wide-angle α-type X-ray pattern, that in its physical properties and to some extent in its amino-acid analysis it resembles myosin—there appears some justification to regard it as a transition from the typically corpuscular class of protein to the truly fibrous.

Actin. As first shown by the Szeged school, actin exists in two states, one relatively non-viscous (G, or globular actin), and one highly thixotropic (F, or fibrous actin). The latter state is induced by neutral salts or slight acidity and can be reversed by dialysis at alkaline pH in presence of adeno-

sine triphosphate (ATP) (Mommaerts, 1952). On no very clear evidence, it was thought that the G-form (as its name implies) was a corpuscular type of protein, which passed to the fibrous form in two steps: first, the aggregation of G-particles into large ellipsoid particles, and secondly, the aggregation of these in a more or less linear manner to give a polymer reminiscent of a string of beads (Rosza, Szent-Györgyi & Wyckoff, 1949). Some electron micrographs have shown a bead-like structure in F-actin and others a completely continuous filament (Jakus & Hall, 1947). The assumption that G-actin itself is corpuscular is not supported at all by viscosity measurements (Tsao, 1953*a*) and an asymmetry of about 12 seems more probable. Moreover, some care should be exercised in accepting that structures seen under the electron microscope are quite representative of those in the hydrated native state, especially with fibrous structures which must undergo

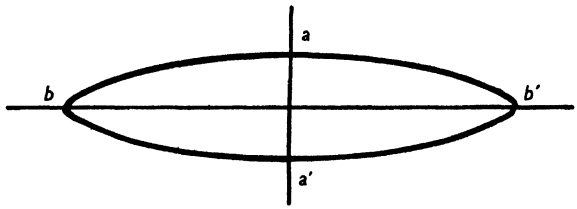

Fig. 2.

tremendous aggregation during drying. Some curious facts about the structure of F-actin have been brought to light by Tsao, and whatever their ultimate interpretation, they are so pertinent to considerations of the structure of protoplasm itself as to deserve a special description.

Tsao (1953*a*) obtained some indication from osmotic pressure measurements that G-actin could itself exist in two forms, monomeric and dimeric, and to explore the transformation in less laborious manner he adopted the polarization-fluorescence method of G. Weber (1952). Fluorescent groups linked to a protein can impart information as to the rotational Brownian movement of molecules; if they are excited, they will emit, after an interval of time characteristic of the attached group, a fluorescent ray. If the turning motion of the molecule to which the group is linked is very fast compared with the lifetime of the excited state, the emitted beam will be completely depolarized. If it is commensurate with it, the emitted beam will be partially polarized. The Brownian motion of an asymmetric molecule such as is represented in Fig. 2 can take place round the long axis bb' and around aa', giving rise to two relaxation times ρ_b and ρ_a respectively. (Actually, three rotations may be present if there are two dissimilar shorter axes.) For proteins up to a particle weight of 300,000 and an axial ratio greater than 10,

the relaxation time ρ_a is much greater than the lifetime of the excited state of the particular fluorescent group employed (1-dimethylaminonaphthalene-5-sulphonyl chloride), and is not measured. If the polarization is measured as a function of temperature, it is possible to deduce a mean harmonic relaxation time ρ_h, which in this example (i.e. for particles of asymmetry greater than 10) is twice ρ_b. Now ρ_b, in its turn, can be related to ρ_0, the relaxation time of a sphere of the same molecular volume; in fact,

$$\rho_0 = \tfrac{3}{4}\rho_b = \tfrac{3}{8}\rho_h = \frac{3\eta\overline{V}}{RT},$$

i.e.

$$\overline{V} = \frac{RT\rho_h}{8\eta},$$

where η is viscosity and $\overline{V}$ the molecular volume.

It is thus possible, for rod-like molecules, to calculate $\overline{V}$ (and hence the particle weight), and if, in addition, the asymmetry is known from other types of experiment, the dimensions of the particle can be ascertained.

Careful measurements of the intrinsic viscosity of a G-actin preparation which by osmotic-pressure measurements possessed a particle weight of 74,000 gave a value indicating for a prolate ellipsoid of revolution an axial ratio of ~ 12. It was thus possible to apply to G-actin the relationships just discussed, and then it became clear that, according to conditions, two values of ρ_h could be found: one tending towards 15×10^{-8} and one towards $30{\cdot}8 \times 10^{-8}$ sec., these actual values being the extremes observed. It will be noted by reference to the above equation that these relaxation times correspond also to particle weights roughly in the ratio 2:1 (70,000 and 140,000), confirming the results of the osmotic-pressure experiments.

Tsao had obtained the impression, by studying what at first were fortuitous conditions, that divalent metal ions were involved in the monomer-dimer transformation of the two types of G-actin. Compounds which combine with Ca or Mg encouraged monomer formation, and in the presence of versenate, a simple change of temperature was sufficient to transform the one type to the other, the dimer existing below 21° and the monomer above. Actin was shown by Straub & Feuer (1950), and since by many workers, to contain either ADP or ATP as prosthetic group, and it is possible that the monomers link together through a divalent metal attached to this group.

Other estimates of the particle weight of actin are not very concordant, but it must be stressed that there is a special difficulty in the case of G-actin; to overcome the Donnan effect or electro-viscous effects many conventional methods require the presence of salt, which as we have seen transforms G-actin to the F-form.

The F-form of actin combines with myosin to give sols of high viscosity, which is instantaneously reduced on addition of small amounts of ATP. If the latter is added, not to a sol, but to an actomyosin gel below its salting-in threshold, a remarkable synaeresis ensues which has been studied intensively by virtue of its relation to the contractile process itself (see Szent-Györgyi, 1951). It is logical at this stage, therefore, to consider the nature of F-actin itself.

F-ACTIN AND ITS INTERACTION WITH MYOSIN AND ATP

The long threads seen under the electron microscope when isolated F-actin is dried have been identified with the apparently continuous filaments which can be seen in the myofibril itself, particularly when much of the myosin has been shed by the Hasselbach-Schneider extraction (Hanson & Huxley, 1953; Hasselbach, 1953). (Actually there is now very good evidence that the continuity of the actin filaments is broken at the *H* space in the middle of the *A* band.) On one occasion at least (Astbury, Perry, Reed & Spark, 1947), a dried F-actin film has also given an X-ray diffraction pattern on which the meridional spacings are similar to those found for dried muscles and living frog sartorius. The fibre period of about 410 Å. is thought to be due to the actin component of the muscle, since isolated myosin has never under any conditions yielded anything more than the wide-angle α-keratin type pattern. The presence of such sharp meridional spacings indicates that the actin system forms long continuous structures which are extremely well ordered and oriented. The adductor muscles of many molluscs contain a diffracting system with a period of 725 Å. (Bear, 1944), and this too is likely to belong to the actin component of what is essentially a smooth muscle. On these several facts, taken in conjunction with the properties of an F-actin sol—its flow birefringence, gel-like character—it seems natural to suppose that G-actin particles join up in a very regular manner, both lengthways and sideways, to give long threads which exist as such in living and dried muscles, and in solutions of isolated F-actin itself.

Tsao's studies of actin by G. Weber's method have introduced a puzzling feature into this simple picture for F-actin (Tsao, 1953*b*), and it is important to discuss it for the bearing it may have on other protoplasmic structures. The dimeric form of actin in the presence of salt passes to the thixotropic state, and if this is really due to rigid polymers, then the rotational Brownian movement in *any* direction should be too large to be measured; yet, in fact, Weber's method records no difference in the relaxation time as between the

non-viscous dimeric state and the thixotropic. It may be argued, of course, that the gel structure is imparted by just a few long filaments amongst which dimeric actin moves freely. This cannot be so because wherever inactive actin is present—i.e. actin which cannot be induced to become thixotropic—it is possible to separate it by centrifuging down the gel portion, and squeezing out in the process the inactive material in the supernatant liquid. Practically all of Tsao's material sediments as a gel.

One way of explaining this curious result would be to suppose that the attached fluorescent groups are exhibiting an internal rotation independent of the rest of the molecule; such movement would need to be restricted, since it makes no difference to the method if they possess a rotation commensurate with the movement of the free dye in solution. However, such an effect has not generally been observed in other proteins. Another explanation could be that the structures seen in muscle and in electron micrographs of actin itself are artifacts of drying, and that in solutions of F-actin no actual filaments exist. In that case, the thixotropy and gel character could be due, not to polymerization in the conventional sense, but a swarming of the molecules in definite areas of solution, orientation being maintained by long-range forces—a phenomenon found in gels of tobacco mosaic viruses. It is only a step further to envisage that although a chemically continuous polymer is not formed, there occurs a kind of polymerization in which the individual links in the filament are free to rotate around the long axis. According to Oncley (private communication), such an effect can be found in the mercury-linked dimer of serum albumin, where a relaxation time of both monomer and dimer can be defined. The properties F-actin possesses in solution—its sedimentation as a gel, its flow birefringence and anomalous viscosity, its high particle weight by light scattering (Mommaerts & Khairallah, 1951)—are not necessarily opposed to the structures proposed, since they reflect only the macroscopic state of the system however it is attained. In particular, what has been considered as true flow birefringence is probably a photoelastic effect, since the well-oriented actin filaments in muscle itself are completely isotropic. What cannot be evaded, however, is the regularity of the diffracting system in muscle, and any final picture of F-actin must take account of that. The fibre period of 410 Å. is undoubtedly due to actin, and is of the same order as Tsao's tentative estimate of the monomer length ($\sim$300 Å.). The equatorial spacings (Huxley, 1953) suggest the existence in the *A* band of filaments 100–150 Å. in width arranged in a hexagonal array 440 Å. apart. These spacings, however, refer only to the myosin component. The actin component, found in the *I* band and part of the *A* band, gives rise to a secondary hexagonal array of filaments which are only 40 Å. in width, and on Tsao's

estimate of the monomer width (25 Å.) could accommodate no more than about four monomers side by side.

The concept of F-actin as an oriented system composed of units with individual freedom of rotation (see G. Weber, 1953) is extremely important for biology, since it might explain 'structure' and gel formation in protoplasm. In the mitochondrion, too, there is much evidence for the organization of whole enzyme systems functioning within a lipid-rich medium and attached such that they resist extraction with water. G. Weber (private communication) has envisaged that they could be fixed in the sense that translational motion is restricted, but free by their rotational motion to interact with substrate or with each other. This idea is most attractive in the case of a chain of respiratory enzymes such as are in fact contained in mitochondria, where the reduced prosthetic group of one member could, during its rotation, interact with the oxidized prosthetic group of the next member in the chain. It is to be hoped that physical chemists will give more thought to such unconventional ideas, and will become attracted to the underworld of long-range forces and pseudo-polymers.

SUBUNITS OF MYOSIN

Whilst nothing is known of the subunits of the actin monomer, it has already been mentioned that the tropomyosin particle of 53,000 cannot be fragmented further. Myosin, however, apart from its crucial role in contraction, is very important for the light it throws on the biogenesis of large protein molecules. It is almost axiomatic, even from the early studies on the seed globulins, haemocyanins and some of the viruses, that really large protein molecules are aggregates of smaller ones; that in the nature of the biosynthetic mechanism there is an upper limit to the length of a peptide chain. From end-group assay, there does not appear to be on record a polypeptide chain of molecular weight greater than 150,000 (in the case of γ-globulin (Porter, 1950)), and even this does not preclude the presence of cyclic chains.

Tsao (1953*c*) has followed the time course of the depolymerization of myosin in neutral urea and guanidine solutions. It was hoped to show not only the presence of subunits, but to explore whether tropomyosin itself formed part of the myosin molecule. It was found that the products of fragmentation could be chemically fractionated in two different ways: either by addition of ethanol to the urea-protein solution, or by salting-out with ammonium sulphate after dialysing away the urea. The fraction precipitating last in both cases was of low molecular weight—about 16,000—and comprised rather less than 10% of the original myosin. The residual

fraction of the myosin, however, was still of high molecular weight, and fragmentation seemed to have been confined to the stripping-off of some polypeptide material from the myosin framework. A more profound fragmentation could be achieved, rather surprisingly, by bringing the pH to 10·7, and then either myosin itself, or its framework, was found to fragment to units of average particle weight 165,000.

Neither *N*-terminal nor *C*-terminal assay give well-defined results for myosin. Traces of several amino-acids appear as *N*-terminal residues, amounting in all to 1 group/500,000 g. myosin (Bailey, 1951). The carboxypeptidase method liberates isoleucine as a *C*-terminal acid—the same incidentally as for tropomyosin—but no more than 1 group/300,000 g. (Locker, 1954). The *N*-terminal acids of the original myosin seem to be associated predominantly with the polypeptide material, whilst the 'framework', by the methods used, appears to contain neither *C*-terminal nor *N*-terminal acids. Thus Tsao obtained the impression that myosin was composed of 4–5 highly asymmetric (possibly cyclic) units of average particle weight 165,000 and 4–5 'open' polypeptide units of average weight 16,000.

A more exciting approach to the substructure of myosin has been gained by the use of enzymes. Gergely (1950) and Perry (1951) independently observed that myosin could be transformed to a water-soluble product (i.e. its globulin properties were largely lost) by the rapid initial action of trypsin, without loss in its ATPase activity. In the ultracentrifuge, the reaction was found to be of the all-or-none type, a rapid splitting into two components, followed by more extensive proteolysis and loss of enzyme activity.

The two components have been separated (A. G. Szent-Györgyi, 1953) and called H- and L-meromyosin, the suffixes signifying heavy and light respectively. Whilst the L-meromyosin is pseudocrystalline and has the properties of a globulin which is readily salted-in, the H-meromyosin is albumin-like and possesses all the ATPase activity of the original myosin. With this go two other properties which have always been linked to the enzyme character in intact myosin, most of the SH groups, and the ability to combine with actin to give an ATP-sensitive complex. H-meromyosin comprises some 57% of the myosin molecule and L-myosin $43 \pm 2\%$, whilst 3% of peptide material not precipitable by trichloracetic acid has not been recovered or characterized.

A. G. Szent-Györgyi has concluded that these two components are the subunits of myosin, with the implication that each is separately synthesized before being built up into the myosin molecule. Whilst this is very probable, it is difficult to draw any final conclusions at present; H-meromyosin could

be the functional part of the molecule split off by proteolysis from the inactive part, just as degradative processes with other proteins have led to the isolation of a biologically active fragment. It would be useful to know how many peptide bonds are broken, and what has happened to Tsao's 'open'-chain polypeptide component. There is no direct evidence for the presence of tropomyosin as an integral part of myosin, and the role of the latter in the fibril is entirely unsolved.

SOME GENERAL CHARACTERISTICS OF THE MUSCLE PROTEINS

For convenience, the more important characteristics of the proteins so far discussed are collected in Tables 1, 2 and 3. Amino-acid analysis (Table 1) shows that tropomyosin contains no less than 45% of its total side-chain groups as charged groups, and of the three proteins, actin is the least polar. Tropomyosin contains neither tryptophan nor proline, and only a very small amount of phenylalanine; myosin is deficient in tryptophan—an unfortunate fact for a major dietary protein. Both myosin and actin contain ~0·9% of sulphydryl groups (calculated as cysteine), which are considered important to their biological function.

The physical data given in Table 2 are not exhaustive. More extensive compilations have been made by Weber & Portzehl (1952) and by Bailey (1954). The data in Table 3 for the meromyosins are abstracted from the paper of A. G. Szent-Györgyi (1953).

THE INTERACTION OF MYOSIN, ACTIN AND ATP, AND ITS BEARING ON CONTRACTION

The present section on the behaviour of actin and myosin in presence of ATP is intended to be no more than a prelude to the more exhaustive contribution of Prof. Weber. The existence of actin was presaged by the observations of the Szeged school (Banga & Szent-Györgyi, 1941–2) and by Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen (1944), who observed that ATP exerted a dramatic effect in diminishing the viscosity and double refraction of flow of 'myosin' sols. Provided they do not interact, the viscosity of a mixture of two proteins in solution should obey the straight-line relation (Kaumans, 1949)

$$\log \eta_{I+II} = \log \eta_I + \log \eta_{II}.$$

In presence of ATP, actin and myosin do, in fact, obey the rule (H. H. Weber, 1950), showing that at ionic strengths above the salting-in threshold for actomyosin, very little non-specific interaction exists. As the ATP is

Table 1. *Some chemical characteristics of muscle proteins (rabbit)*

	As % of total groups			Residues /10^5 g. of protein				End-groups	
	Free acid	Base	Polar groups	Tryptophan	Proline	Phenylalanine	SH as cysteine	*N*-terminal	*C*-terminal
Tropomyosin	26·6	18·4	63	0	0[a]	4[a]	nil	nil[b]	Isoleucine[c]
Actin	12·9	12·0	49	10[a]	44[a]	30[a]	7[d]	nil[c]	Phenylalanine[c]
Myosin	18·0	16·1	57	4	22[a]	26[a]	7[d]	Small amounts[b]	Small amounts[c]

[a] Values of Kominz, Laki and Hough by the Moore-Stein chromatographic method (personally communicated); [b] Bailey (1951); [c] Locker (1954); [d] Tsao & Bailey (1953); the rest by Bailey (1948*b*).

Table 2. *Some physical characteristics of muscle proteins (rabbit)*

	Tropomyosin	Actin (G-form)	Myosin
Sedimentation constant ($s^0_{20} \times 10^{13}$)	2·6[a], c=0·6	2·7–4[b, c]	6·7[d], 7·1[e], 6·7[f]
Diffusion constant ($D^0_{20} \times 10^7$)	2·4[a], c=0·6	2·5[b, c]	0·9[g]
Particle weight:			
s and D	—	150,000[b, c] (dimer?)	850,000[g]
Light scattering	55,000[h]	57,000[i] 120,000[c] 80,000[j]	—
Osmotic pressure	53,000[k]	74,000[i] (monomer) 140,000[i] (dimer)	840,000[g]
Polarization-fluorescence	—	70,000[i] (monomer) 140,000[i] (dimer)	—
Intrinsic viscosity	0·523[k]	0·21[i] (monomer) 0·5[i] (dimer)	—
Rotational relaxation time $\rho_h \times 10^8$ sec. at 25°	8[k]	15[i] (monomer) 30·5[i] (dimer	—
Length (Å.)	~400[k]	~290[i] (monomer) ~580[i] (dimer)	2,300[g] (from f/f_0) 1,500[m] (light scattering)
Thickness	~15[k]	~25[i] (monomer)	25[g] (from f/f_0)

[a] Bailey, K., Gutfreund, H. & Ogston, A. G. (1948). *Biochem. J.* **43**, 279.
[b] Snellman, O., Erdös, T. & Tenow, M. (1947). *Proc. 6th Int. Congr. Cytol. Stockholm*, p. 247.
[c] Johnson, P. & Landolt, R. (1951). Size and shape factor in colloidal systems. *Disc. Faraday Soc.* p. 179.
[d] Mommaerts, W. F. H. M. & Parrish, R. G. (1951). *J. Biol. Chem.* **88**, 545.
[e] Portzehl, H., Schramm, G. & Weber, H. H. (1950). *Z. Naturforsch.* **5***b*, 61.
[f] Johnson, P. & Landolt, R. (1950). *Nature, Lond.*, **165**, 430.
[g] Portzehl, H. (1950). *Z. Naturforsch.* **5**, 75.
[h] Doty, P. & Sanders (private communication).
[i] Mommaerts, W. F. H. M. (1952). *J. Biol. Chem.* **198**, 445.
[j] Steiner, R. F., Laki, K. & Spicer, S. (1952). *J. Polym. Sci.* **1**, 23.
[k] Tsao, T.-C., Bailey, K. & Adair, G. S. (1951). *Biochem. J.* **49**, 27.
[l] Tsao, T.-C. (1953). *Biochim. biophys. Acta*, **11**, 227.
[m] Mommaerts, W. F. H. M. (1951). *J. Biol. Chem.* **188**, 553.

Table 3. *Physical data on the meromyosins**

	s^0_{20}	D^0_{20}	M	f/f_0	Suggested length and diameter (Å.)
H-meromyosin	6·96	2·91	232,000	1·78	430 × 30
L-meromyosin	2·86	2·87	96,000	2·45	550 × 17

* Szent-Györgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.

hydrolysed, however, the viscosity rises to a very high value if the proportions of myosin and actin are optimal (Fig. 3).

Godeaux, unknown to later workers, had obtained a clue in 1944 as to the role of SH groups in the interaction, showing that actomyosin gel, treated with SH reagents no longer synaeresed in presence of ATP. Independently, Bailey & Perry (1947) treated myosin with various SH reagents (oxidants, organic mercurials, alkylating reagents) and showed that as the ATPase activity of the myosin declined, its ability to complex with actin declined too in a parallel manner, and hand in hand with these effects there

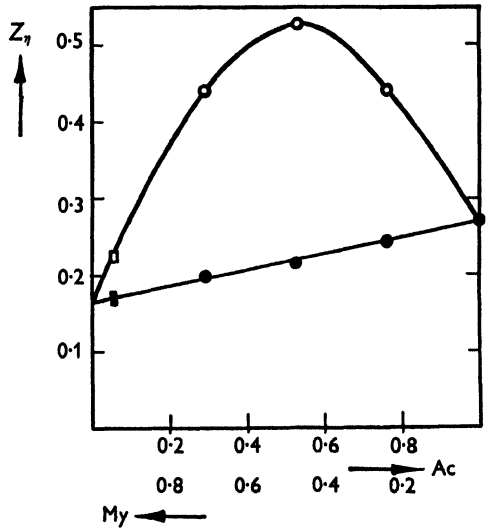

Fig. 3. Viscosity changes in actomyosin (from Hasselbach, 1953). The myosin was prepared from isolated fibrils in rigor and added to pure F-actin. Top curve without ATP, bottom curve with ATP.

$$Z_\eta = \frac{\eta_{sp}}{C_{c=0}}, \quad \text{where} \quad C \text{ is g./l.}$$

was a progressive blocking of the reactive SH groups of myosin. The converse experiment, the reaction of the SH groups of actin, has very little effect on the interaction. The idea was gained from these experiments that ATP and actin each combine at the same active centres of the myosin and that ATP was able to displace actin by virtue of its greater affinity. Mommaerts (1950) has estimated that only 1 g.-mol. of ATP is required for 100,000–300,000 g. of myosin. With the demonstration (Straub & Feuer, 1950) that actin contains a nucleotide prosthetic group, it is tempting to suppose that this is the combining point for the myosin.

It is a characteristic of many enzymes that the SH groups of cysteine side-chains are essential to their activity, and the author (Bailey, 1946) has considered it significant that wherever ATP acts as substrate, it does so

with enzymes 'either of proven or suggested SH character'. There is still no need to modify this assertion, but the role of SH groups is quite unknown. Acting in conjunction with other types of side-chain groups, they may simply form some special configuration receptive to the ATP molecule. It is, however, significant that where their exact role has been elucidated for certain kinds of coenzyme they appear to participate in a chemical reaction, e.g. the pyrophosphorylation of coenzyme A, the additive reaction of phosphoglyceraldehyde to glutathione in the oxidative step of glycolysis.

In the gel state which exists at a reduced salt concentration (ionic strength 0·25), the action of ATP on actomyosin is very different, giving rise to an intense synaeresis. If this could be explained in molecular terms, one might be very near to an explanation of contraction itself. First, however, we should note that the water changes in themselves may have nothing to do with the physiological process. In the gel system, the change induced by ATP, whatever its exact nature, is acting against the non-specific interaction of the two proteins, and the entire system must shrink because the particles are randomized; in solution, these interactions are absent, and the modification of the specific interactions caused by ATP are the last barrier, so to speak, to a change in the physical state of the system, resulting in large viscosity effects. It is difficult to extrapolate to muscle itself, but it seems likely that the overall ionic strength in muscle ($\sim$0·2) is sufficient to minimize non-specific interaction, so that the specific effects of ATP are preeminent. As Prof. Weber will develop from his own work with model systems (see Weber & Portzehl, 1952), and from the work of the author and Marsh (Bailey & Marsh, 1952; Marsh, 1952), ATP has a duality of action which can explain not only the differences between resting and contracted muscle, but also differences between various types of muscle. Contractile phenomena are always associated with the *splitting* of ATP, and relaxation with the *presence* of ATP. These two effects are geared in such a way that relaxation will predominate unless actin and myosin are 'combined'; but the very same concentration of ATP which causes contraction may cause relaxation if splitting is inhibited. In resting muscle, this is achieved by the presence of a non-dialysable factor (Marsh, 1952) which Bendall (1954) concludes to be myokinase, so that the typical plasticity of relaxed muscle arises because ATP can exert its 'dissociating' effect. When the inhibition is removed, by stimulation, the increased breakdown of ATP causes both combination and subsequent contraction; the faster the breakdown, the faster the contraction.

So much was clear when the assignation of definite proteins to definite sites in the myofibril was largely guesswork. Now, however, it seems certain from the elegant work of A. F. Huxley & Niedergerke (1954), and of H. E.

Huxley & Hanson (1954), that the *A* bands remain of constant length both in passive stretch and in contraction; in the latter state the *I* filaments of actin are drawn into the *A* bands, so that at 65% of the resting length adjacent *A* bands run together at the *Z* line.

At this point we may ask two vital questions: (*a*) what is the molecular significance of this reaction? and (*b*) what is the nature of the energetic coupling to the actomyosin system?

As concerns (*a*), it has always seemed likely, from the properties of myosin itself and its ability *in vitro* to transform from the α to the β state, or to the supercontracted state, that the ultimate cause of contraction lay in some change of intramolecular folding in the myosin component (Astbury, 1947). There are, however, two very strong reasons why we shall need to give much more weight to the role of actin, which so far has been given an ancillary and ambiguous role. First, if one must accept that myosin, as an α-type protein, conforms with the Pauling-Corey model, in which all H-bonds are intramolecular, it is difficult to see how any reaction with ATP, whether one of adsorption (Morales & Botts, 1953) or of topochemical nature (Riseman & Kirkwood, 1948), could break and reform them as the molecule passes reversibly from one configuration to another. Secondly, the constancy of length of the *A* band, and thus of the myosin filaments, leads to the view, already expressed by Huxley & Hanson (1954), that molecular changes are primarily associated with the actin particles as they are drawn into, and fold inside, the *A* band.

Somewhat in line with this trend of thought are Tsao's (1953*b*) results in which the fluorescence-polarization method was extended to a study of the behaviour of labelled actin in presence of myosin and ATP. It will be recalled that the thixotropic form of actin behaves kinetically as though it were composed of dimeric molecules. When myosin is added to it, there is no evidence from the resulting depolarization that an anastomosed complex of actin and myosin is formed; indeed, the rotational relaxation time changes at once to that of the monomer, and on addition of ATP, back to that of dimer. The change is freely reversible as the ATP is split by the myosin ATPase.

What these changes signify in molecular terms is difficult to say, especially when, in contradiction to all the macroscopic properties of the actomyosin system, they seem to indicate that there is no complex formation between actin and myosin. (Actually, a rather similar conclusion has been reached by Blum & Morales (1953) from light-scattering studies.) For the first time, however, they have indicated what appears to be an instantaneous change in the actin component actuated by myosin and by ATP.

With respect to (*b*), and in keeping with the general belief that energy must be transmitted to the *myosin* component for contraction to occur, there

have been several hypotheses as to how this might be achieved; by phosphorylation of aliphatic hydroxyl side-chains, an exothermic adsorption of the ATP molecule, and, to these could be added, by analogy with known reactions, phosphorylation of a sulphydryl, amidine or carboxyl group. All these postulates explain contraction as an intramolecular change in the myosin filaments induced by a change of charge, according to the historic concept of K. H. Meyer (1929). They all neglect also the role of actin.

Some evidence has been adduced (Straub & Feuer, 1950; Mommaerts, 1952) that the transformation of G- to F-actin is accompanied by the conversion of ATP to ADP. If a molecular change in actin is really important in contraction, and if Tsao's findings are relevant to it, then one might imagine the myosin ATPase as directing it, and extraneous phosphorylating processes as restoring it, e.g.

$$\text{ATP-actin} \xrightarrow{\text{myosin ATPase}} \text{ADP-actin} + \text{phosphate},$$

$$\text{ADP-actin} + \text{ATP} \xrightarrow{\text{phosphorylation}} \text{ATP-actin} + \text{ADP}.$$

Unfortunately for such a hypothesis, Perry (1954) has been unable to obtain any evidence that the creatine phosphokinase system, for example, is able to phosphorylate the fixed ADP of the isolated myofibril.

In spite of all these considerations, some recent work from Engelhardt's school in Russia focuses once more upon myosin as the essential agent of contraction. Actin-free myosin stained with various dyes will undergo a synaeresis with ATP (Ashmarin, 1953), suggesting that the phenomenon is consequent upon a charge modification brought about physiologically by actin. In line with this work, Kafiani & Engelhardt (1953) find that fibres of myosin prepared from surface films by Hayashi's method (1952) will contract anisodimensionally in presence of ATP at pH 9. These authors suggest that myosin contracts at the optimal pH of the myosin-ATPase which is 9 in absence of actin and 7 in its presence. This concept deserves very serious consideration. Under conditions of physiological pH it would explain why myosin needs to be combined with actin before contraction is elicited; why also heat output of a muscle is proportional to shortening, since overall shortening is proportional to the number of sites at which actin and myosin are interacting; in other words, to the number of active ATPase centres.

We must finally admit, despite some promising trails, that we are ignorant of the molecular details of contractility. It seems more important at the present juncture to decide in the light of the recent findings of the English and Russian schools whether molecular changes, whatever their nature, are associated with the actin, the myosin, or both.

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THE COMPONENTS OF THE MYOFIBRIL AND THEIR RELATION TO ITS STRUCTURE AND FUNCTION

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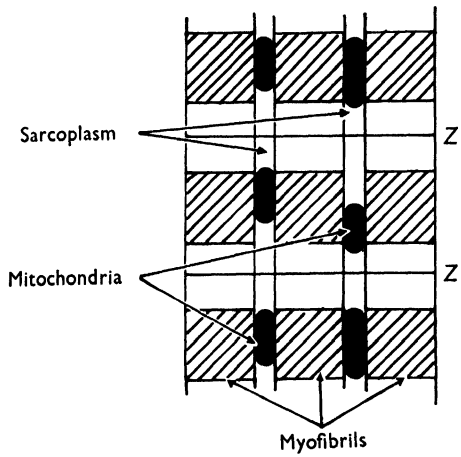
The existence of longitudinal elements within the cells of both smooth and striated muscle tissue has been known since the work of Schwann (1839) and of Bowman (1840), but it was some years later before Kölliker (1851) suggested that these structures, the myofibrils, were the contractile units. To-day it is widely accepted that the myofibrils constitute the contractile apparatus of the muscle fibre, yet from time to time evidence has been produced for the existence of contractile properties in the sarcoplasm itself. With mature muscle cells there appears to be little doubt that contractile function is restricted to the myofibrils. Nevertheless, in view of the observations with primitive muscle cells such as tissue-culture myoblasts which show contraction even though myofibrils cannot be detected (de Renyi & Hogue, 1938) and the general contractile properties of undifferentiated cytoplasm, it appears that the contractile property of the myofibril is simply a highly specialized development of a general function of protoplasm. Indeed, recently evidence has been provided by Hoffman-Berling (1953) and Hoffman-Berling & Weber (1953) for a wide distribution in cells, other than those from muscle, of systems capable of shortening in the presence of adenosinetriphosphate (ATP) in a manner similar to actomyosin itself.

ARRANGEMENT OF MYOFIBRILS IN THE CELL

Individual myofibrils can be readily demonstrated after fixation but are not easily seen in living muscle, and consequently it has been suggested that these structures are artifacts which do not exist as such in living muscle. Controversy on this point has existed since Bowman's early observations, and recently Szent-Györgyi (1953) has concluded from the results of his electron-microscope investigations of muscle cross-sections (Morgan, Rozsa, Szent-Györgyi & Wyckoff, 1950) that the cytoplasm consists of a continuous mass of protofibrils (filaments) which break up along cleavage planes into columns which 'have often been erroneously interpreted as preformed "fibrils"'. In muscles such as the psoas of the rabbit which are relatively poor in sarcosomes, the myofibrils lie close together and are not

readily distinguished in longitudinal section, but clear evidence for spacing between the myofibrils is provided in muscles of high oxidative activity such as pigeon breast, heart and insect flight muscles which contain large numbers of sarcosomes. These granules lie between the myofibrils, separating them and often extending along the length of one or more bands (see Text-fig. 1). In some muscles certain of the sarcosomes seem to be arranged in a regular pattern, the lateral spacing being such as to suggest that they are associated with the myofibrillar unit. Further strong evidence for the intracellular existence of the myofibril is given by the fact that when



Text-fig. 1. Diagram illustrating the relation of the myofibrils to the mitochondria and sarcoplasm. The mitochondria (A granules) are represented as relatively large and abundant as they occur in muscles of high oxidative activity, e.g. pigeon breast muscle. I granules are not shown but can be demonstrated, particularly in insect muscle, lying in the sarcoplasm near to the I bands.

muscle is broken up mechanically at physiological pH in dilute salt solutions which have little or no solvent action on the myofibril, it breaks down into structures which are found by electron-microscope examination to be of diameter 1–2 mμ and which are often assembled together in loose bundles of several such units. Myofibrils of diameter less than 1–2 mμ are rarely seen; when they are it is usually apparent that the myofibrillar structure has disintegrated. Much of the other evidence for the existence of the myofibril in the living cell has been admirably presented by Barer (1948).

In the living cell individual myofibrils are arranged alongside one another with great precision, and as Pl. 1, fig. 1 shows it is only when the bands in adjacent myofibrils come out of alignment that the myofibrils can be recognized as individual units. The most satisfactory picture of muscle cytoplasm is one of individual myofibrils closely arranged in parallel array with all the

bands precisely aligned, and in some cases running continuously across the cell, so that each myofibril is surrounded and separated from its neighbour by the soluble sarcoplasm and the sarcosomes (Text-fig. 1).

The resolving power of the light microscope imposes definite limitations on its use for the elucidation of the fine structure of the myofibril, but nevertheless before application of the high-resolution electron microscope to this problem histologists had already described the cross-striated features of skeletal muscle in a detail which even to-day has been little improved upon. The intracellular fibrils of smooth muscle are conspicuous by their lack of features when examined in the light microscope, and it is unfortunate that as yet few electron-microscope studies on this tissue have been published. On the other hand, a great deal is now known about the fine structure of the striated myofibril, and naturally this knowledge has considerable bearing on the formulation of theories of the mechanism of the contractile process. Without a knowledge of the fine structure of smooth muscle, an essentially simpler contractile tissue, it is not yet possible to say which features of the striated myofibril are essential for contraction as a general process and which are merely extra refinements in structure to enable striated muscle to carry out its own specialized type of contractile function. In this communication discussion will be concerned mainly with the myofibril from skeletal muscle.

For a given tissue myofibrils appear to be remarkably uniform in diameter, the precise value obtained depending on the source of the muscle and the method of fixation or preparation employed. The values given for the diameter of a single myofibril from electron-microscope investigations vary from 0·5 to 2·0 mμ, but these values correspond to the width of the ribbon obtained when the myofibril dries down flat on the specimen grid, and the diameter *in situ* will be considerably less. Seen in cross-section after fixation the rabbit psoas myofibrils appear rather rhomboid in outline and about 0·5 mμ in diameter (Huxley, 1953).

In the cell the myofibrils are usually separated from each other by thin layers of sarcoplasm and grouped in bundles which, for example, in breast muscle of the domestic fowl may consist of 4–20 myofibrils (Bennett & Porter, 1953). These bundles are sometimes called muscle columns, and in cross-section they give rise to the so-called fields of Cohnheim. Occasionally myofibrils and muscle columns branch and individual myofibrils or groups of them sometimes switch from one column to another.

CROSS-STRIATIONS OF THE MYOFIBRIL

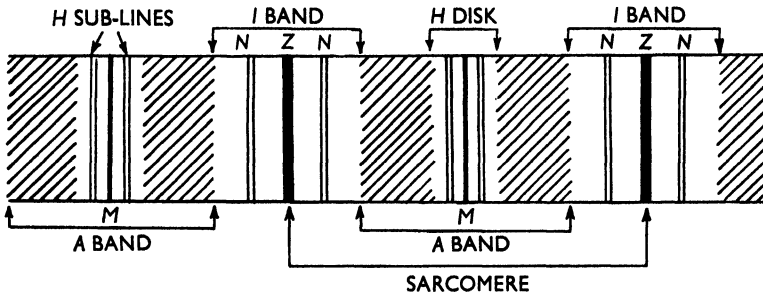
The characteristic feature of skeletal muscle is its cross-striated appearance due to the alternation of the so-called *I* (isotropic) and *A* (anisotropic) bands. When examined microscopically with ordinary light the *A* band appears darker than the *I* band, whereas when polarized light is used the appearance is reversed: the *A* band appears bright because of its birefringent properties and the *I* band remains dark.

Histologists of the last century described a number of other striations in both *I* and *A* bands, and so far the electron-microscope investigations have for the most part provided little more than confirmation of these findings.

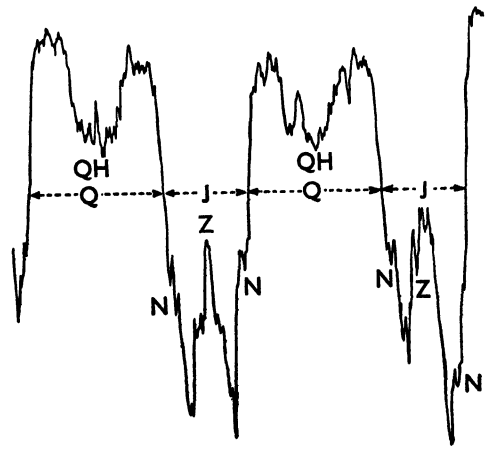
Bisecting the *I* band is the *Z* band or line (telophragma), a readily recognized feature which is taken as one of the fixed points of the myofibril. A convenient unit of myofibrillar structure is the sarcomere which consists of an *A* band bounded on either side by half an *I* band, i.e. that part of the myofibril bounded by two consecutive *Z* bands (see Text-fig. 2*a*). At rest length in the frog the *A* band is 1·4–1·5 μ and the sarcomere length 2·5 μ (Huxley & Niedergerke, 1954). Densitometric tracings of electron micrographs (Bennett & Porter, 1953) indicate that at the *Z* band the amount of osmium-stained material present is much greater than that in the adjacent *I* band area and it corresponds in intensity to that found in the *A* band (Text-fig. 2*b*). A similar distribution of material was indicated earlier by Draper & Hodge (1949) from the shadow outlines of platinum-shadowed toad myofibril preparations. At one time the *Z* band was considered to consist of collagen, but the electron microscope has provided no evidence for the characteristic banded collagen structure, and analysis of isolated myofibrils (Perry, 1953) indicates the absence of protein with collagen solubility characteristics in these structures. The *Z* band runs continuously across the whole fibre and there is evidence that ultimately it is attached to the sarcolemma. When the muscle cell is broken up into myofibrils the *Z* band breaks and can frequently be seen protruding laterally from single myofibrils (Meigs, 1932; Draper & Hodge, 1949). The latter authors were able to demonstrate that in the toad, *Bufo marinus*, the *Z* band consisted of two main sub-bands of 450 Å. width in between which were three symmetrically placed finer bands each of 100 Å. width.

Two less frequently observed minor structures, the *E* and *N* disks, are also found in the *I* band. The *N* disk or band, 'Nebenschiebe' of Engelmann (1878), is located on either side of the *Z* line approximately half-way between it and the *A* band. In some striated muscles, particularly those from insects, granules lie in the sarcoplasm close to the myofibril at the *N* line (*I* granules). For this reason a number of workers have considered

the *N* disk to be an artifact resulting from these particles overlying the myofibril. It appears clear from electron-microscope studies, however, that cross-striations are present in the myofibril itself, at this point. *N* lines have not been reported as invariably present by all workers, but it is likely that they are regular features of skeletal myofibrils from all species as they have been observed in the frog and the rabbit (Barer, 1948), the fowl



Text-fig. 2*a*. Cross striations of the myofibril from skeletal muscle.



Text-fig. 2*b*. Densitometric tracing of a relaxed myofibril from fowl breast muscle. The *A* and *I* bands are indicated by Q and J respectively. (From Bennett & Porter, 1953.)

(Bennett & Porter, 1953) and the toad (Draper & Hodge, 1949). The latter workers have demonstrated this disk to consist of two rather closely spaced filaments in toad muscle.

Separating the *N* disk and the *Z* line is the light band known as the *E* disk (Rollet, 1885). Apparently little is known about this band, although recently Bennett & Porter (1953) have identified it in breast muscle from the fowl. No definite structure has as yet been ascribed to the *E* band, and it is perhaps best considered as the area between the more intensely staining *Z* and *N* lines containing the longitudinal *I* filaments only.

The other fixed point of the myofibril is the *M* line (mesophragma) which bisects the *A* band and forms the central feature of the *H* (Hensens) disk. When present the *H* disk appears as a lighter area in the centre of the *A* band, usually occupying about one-third of that band, and in general there is much greater contrast between the *A* and *I* bands than between the *A* band and *H* disk. The exact relation of the *H* band to the state of contraction is not clear, but according to Hall, Jakus & Schmitt (1946) its presence depends on whether fixation takes place during isometric or isotonic contraction. The latter authors observed marked *H* bands in myofibrils fixed during isometric contraction, whereas in some preparations of isotonically contracted rabbit muscle there was little evidence of the *H* band. According to Jordan (1933) the *H* and *M* bands are not visible when muscle is in the resting state. With preparations of isolated rabbit myofibrils which have rather short *I* bands due to the partial contraction occurring during isolation (Perry & Horne, 1952), the marked features of the *A* band are the *M* and *H* sub-lines. The *H* sub-lines which are striations less regularly observed in the *H* disk on either side of the *M* line, have also been reported by Hall *et al.* (1946) and Barer (1948) in rabbit muscle, and in the toad by Draper & Hodge (1949). The latter investigators showed this system to consist of two fine parallel striations on each side of the *M* lines. In myofibrils isolated from rabbit skeletal muscle in the unfixed condition before preparation for the electron microscope, the *H* sub-line system appeared as a broad single line on either side of the *M* line (Perry & Horne, 1952). This *M* line and *H* sub-line system dominated the H space and was the most marked feature in myofibrils isolated from rigor mortis muscle. The *H* sub-line system is not consistently observed, and it is possible that it may be fairly readily washed away during the preparation of the myofibrils. Philpott & Szent-Györgyi (1932) have recently described additional bands which appear on either side of the *H* space when rabbit psoas muscle at equilibrium length is stretched. These features, rather confusingly named *E* bands in view of the earlier described *E* disks of Rollet, account for 50% of the increase in length during stretch and are considered to be the site of localization of the series elastic component of muscle (Levin & Wyman, 1927). *E* bands also apppear during isometric contraction, and it may well be that the area of the *A* band previously described as the *H* space by other workers has included the *E* bands of Philpott & Szent-Györgyi. Certainly in some of the electron micrographs published by these authors the junction of the *E* and *H* bands closely resembles that which has been described as the *H* sub-line system. It is clear that much work has yet to be done to relate the appearance of the various cross striations to definite phases in the contraction-relaxation cycle.

THE FILAMENTOUS STRUCTURE OF THE MYOFIBRIL

The real contribution made by the electron microscope to our knowledge of muscle structure has been the demonstration that each myofibril is built up of fine protein filaments running parallel to the axis. These filaments, myofilaments or protofibrils as they have variously been called, were considered to run continuously through the *A* and *I* bands, but recent studies on cross-sectioned muscle (Huxley, 1953) with improved techniques have shown that several different kinds of filaments exist in the myofibril, and that those seen in earlier studies with longitudinal sections were in fact composite structures formed by lateral association of these ultimate units. Huxley (1953) concludes that the *A* band contains two sets of filaments each lying parallel to the fibril axis. The filaments of the first set are continuous throughout the *A* band and have a diameter of 110 Å. except over the region of the *H* disk where the diameter is 140 Å. These *A* band filaments form a very regular hexagonal array within which and extending from the edge of the *A* and to the beginning of the *H* disk lie thinner filaments of 40 Å. diameter. Each thin filament lies equidistant from three of the larger primary filaments. In the *I* band are thin filaments only, which Huxley considers are probably continuous with the thin filaments of the *A*, although the experimental evidence does not allow this to be concluded with certainty.

This investigation clearly demonstrates that the myofibril is evenly packed with filaments and is not a tube within the walls of which the filaments are concentrated as the investigations of Pease & Baker (1949) and Draper & Hodge (1949) seemed to indicate. Huxley finds the distance from centre to centre of adjacent thick filaments to be 200–300 Å. from electron microscope studies, whereas his X-ray investigations indicated a value of 440 Å. for this distance. Whichever is the real value, the packing is fairly loose and explains why during preparation of electron-microscope specimens the myofibril dries down to a thin ribbon apparently only very few filaments thick.

The filaments show a marked periodicity along their axis which, although not always obvious when single filaments are examined, is quite striking in the myofibril where the filaments are aligned with such longitudinal and lateral precision that the periodicity can be seen running across the whole myofibril to give the appearance of fine cross-striations. For frog, rabbit and toad muscle the length of the longitudinal repeating unit has been reported as 400 Å. (Hall *et al.* 1946; Draper & Hodge, 1949; and Morgan *et al.* 1950). This value appears to be subject to variation according to the state of the myofibril, for Draper & Hodge (1949) found values ranging

from 250 to 450 Å. the actual value being in fair correspondence to the sarcomere length. Values of 250–300 Å. have also been observed in relaxed muscle, however, and whereas Morgan *et al.* (1950) sometimes observed in isolated filaments or in clear spaces of the myofibril this smaller periodicity as well as the 400 Å. unit, Hoffmann-Berling & Kausche (1950) could only find evidence for a 230–300 Å. unit. On the other hand, Farrant & Mercer (1952) were unable to detect a regular periodicity in the filaments of insect or crab muscle, although a less obvious and finer nodulation could sometimes be seen in the filaments of insect muscle after enzymic digestion.

From time to time finer periodicities have been observed; for example, Bennett (1949) produced evidence of a spiral structure in the filament with a periodicity of 70 Å. Some confirmation of an ultra-structure to the filament is provided by Huxley (1953) who has observed, most clearly in the *H* disk, cross-striations spaced at 50–60 Å. on the thicker of the two types of filaments he has described. Sometimes the finer striations were not seen but an indistinct pattern of dots was present instead, and Huxley suggests that the striations may have arisen by a blurring of this dot pattern. Also Philpott & Szent-Györgyi (1953) have reported a spiral structure of 80 Å. periodicity in the *E* band, the elastic segment. It is very possible that an ultra-structure exists in the myofilament, for at this level we are approaching the dimensions of the protein molecules themselves, but unfortunately the resolution of the electron microscope is not yet great enough to throw light on this problem.

Crab leg-muscle filaments stained with phosphomolybdic acid appear to consist of a central core of diameter 160 Å. surrounded by a more densely stained sheath 40 Å. thick (Farrant & Mercer, 1952). A somewhat similar result was obtained by Huxley (1953) with rabbit muscle, where the 140 Å. thick filaments of the *H* disk appeared in cross-section to be hollow or at least less densely stained in the centre. Huxley is of the opinion that this appearance is an artifact due to incomplete penetration of the stain, but considering the diameter of the filament this seems unlikely. Indeed, Farrant & Mercer (1952) found the tubular appearance equally well defined whether staining was carried out for 5 min. or for several weeks. This observation is clearly of some importance, for it seems to be a definite indication of the composite nature of the thicker filaments and obviously must be considered in attempts to decide the precise location of the myofibrillar proteins.

LOCALIZATION OF THE CHEMICAL COMPONENTS

It is apparent from the foregoing description that some eight or nine banded features can be distinguished by their particular affinities for histological and electron stains, or in some cases by their relative optical densities. It is probable that each of these bands indicates the localization of some specific component which in most cases is likely to be protein, for there is little doubt that the myofibril isolated with its banded structure intact and washed free from sarcoplasm consists mainly of this substance. When isolated free from sarcoplasm the myofibrils contain 4–7% fat-soluble material, some of which is probably picked up during the preparation; ash and nucleic acid make up together less than 1%, whereas the total nitrogen content is 14·1% (see Table 1).

Table 1. *Composition of myofibrils isolated from rabbit skeletal muscle*

Component	% dry wt.	Method of isolation and medium used	Remarks
Total N	14·1	0·078 M-borate, pH 7·1 collagenase	—
Ether-alcohol extractable material	4–7	0·078 M-borate, pH 7·1 collagenase	—
Ash	0·5	0·078 M-borate, pH 7·1 collagenase	—
Total P	0·1–0·5	0·078 M-borate, pH 7·1 collagenase	—
Total nucleic acid and phosphoprotein P*	0·04–0·05	0·078 M-borate, pH 7·1, collagenase, mechanical	—
Labile P (10 min. at 100° in N-HCl)	0·011	0·078 M-borate, pH 7·1, mechanical	Represents 2·7 μmoles/g. ADP, 0·47 μmole/g. ATP
Tropomyosin	4	0·078 borate, pH 7·1, mechanical	—
Borate-soluble unidentified component	9	0·078 M-borate, pH 7·1, mechanical	—
LiCl-insoluble fraction	9	0·078 M-borate, pH 7·1, mechanical	—
Actin	23	—	From Hasselbach & Schneider (1951) assuming myofibril contains 65% of total muscle protein
Myosin	55	—	Estimated by difference

* Schmidt & Tannhauser (1945).

Some of the banded structures may arise simply from modification of the electron scattering and general staining properties by a concentration of inorganic material at the particular band. Evidence that inorganic material is not distributed regularly along the myofibril has been provided by micro-incineration techniques, as, for example, in the studies of Scott (1932) and

Scott & Packer (1939), who found a concentration of ash in the *A* and *Z* bands. This finding does not necessarily imply that a relatively high concentration of minerals is associated with the proteins of the *A* and *Z* bands, for the greater density of the myofibril at these points must be mainly due to a concentration of protein, the associated ions of which (gegenions) would naturally give rise to an increased amount of ash on incineration. These results, taken with those of Draper & Hodge (1950), do suggest, however, a differential localization of inorganic elements. In the latter study micro-incineration was carried out in the electron beam, conditions under which K and Na salts are volatilized away, whereas Ca, Mg, Fe and P are not volatile and constitute the main components of the remaining ash. After this treatment there was an overall loss of contrast between the *A* and *I* bands, but more striking was the fact that the *Z* and *M* bands had disappeared altogether and the *H* sub-lines were the most marked features of the incinerated myofibril. Filaments could no longer be recognized after incineration, but the ash was distributed along the axis with a marked periodicity of 250–450 Å., corresponding to that observed in the original filament. It is difficult to apply a rigid test to confirm that the method used by Draper & Hodge does satisfactorily vaporize all the protein carbon under the high vacuum conditions in which it is carried out, and that the remaining electron scattering material consists simply of the non-volatile elements. With this reservation it can be concluded that there is considerable concentration of Ca, Mg and Fe at the *H* sub-lines, whereas the *Z* and *M* lines are particularly low in these elements and that they are largely protein in nature.

Undoubtedly the majority of banded features consist of protein, but much has yet to be done to decide how far each structure is due to a different protein or to the concentration of a particular protein which is already present elsewhere in the myofibril. So far only three proteins, myosin, actin and tropomyosin, have been well characterized as components of the myofibril. These proteins were assigned to the myofibril on the basis of their properties long before direct confirmation of their intracellular localization was available, but recent studies on the isolated myofibrils (Perry, 1953; Hasselbach, 1953) have provided direct justification for these assumptions, and as yet there is no evidence that in the skeletal muscle cell these three proteins are to be found other than in the myofibril.

Investigation of the isolated myofibrils from rabbit skeletal muscle has indicated the presence of other less well-defined proteins. The existence in the myofibril of proteins other than the three already named might be expected, for although actin and myosin probably are the main constituents of the longitudinal filaments it is likely that some of the cross-bands, for

example, the *Z* and *M* lines, are built up of other proteins in view of their differences in form and function from the filaments. Certainly the myofibril is not completely soluble under conditions when all the actomyosin might be expected to go into solution. Salt concentrations of ionic strength greater than 0·5 extract extremely viscous solutions of actomyosin which readily respond to ATP with a considerable fall in viscosity, and after such extraction a ghost or residue of the myofibril, which can be centrifuged down, is left behind. For example, after exhaustive extraction with 0·90 M-LiCl, which removes all actin and myosin, 8–13 % of the total protein nitrogen remains behind as an insoluble residue containing 16·7 % total nitrogen and 0·14 % total phosphorus. Myosin ATPase distribution studies indicate that LiCl extracts all the myosin, and it is unlikely that the residue contains actin for the following reasons:

(1) Actin cannot be prepared from this residue by applying the Straub (1942) procedure.

(2) LiCl extracts completely the bound nucleotide of the myofibril (see p. 222).

(3) LiCl is an extremely efficient extractant of actomyosin from minced muscle containing whole cell fragments (cf. Bate-Smith, 1937), and it would be expected to be at least equally efficient with a myofibril suspension. This residue left after exhaustive extraction with LiCl probably consists of insoluble protein from the cross banded structures although the possibility that it may in part have come from the filaments themselves cannot be excluded.

Extraction of the myofibril with more alkaline reagents such as Weber's solution or with potassium chloride solutions containing potassium iodide brings approximately 97 % of the protein nitrogen into solution.

At lower salt concentrations (ionic strength 0·3–0·5, depending on the conditions), with the addition of 0·01 M-pyrophosphate to aid the dissociation of actomyosin, myosin can be selectively extracted from the myofibril. Differences exist between the extractability of the fibrous proteins from whole minced muscle and from isolated myofibrils due possibly to differences in the accessibility of the myofibrillar protein to the extractant and the modifying effects of the sarcoplasm on the extraction. Whereas L-myosin can be selectively extracted from fresh minced muscle by solutions containing 0·48 M-KCl, 0·05 M-phosphate and 0·01 M-pyrophosphate pH 6·0–6·5 (Hasselbach & Schneider, 1951), this medium extracts actomyosin rich in actin from myofibrils if they are isolated from fresh muscle (Perry, 1953). If, however, the muscle is allowed to go into rigor before preparing the myofibrils, viscosity studies show that the Hasselbach & Schneider medium then selectively extracts L-myosin (Hasselbach, 1953). Under these con-

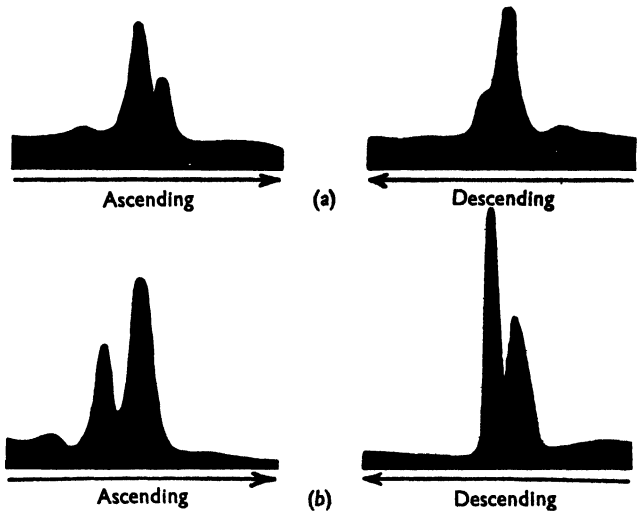
ditions the *A* band substance disappears and the latter band becomes indistinguishable from the *I*. On exhaustive extraction the *Z* band also disappears, but Hasselbach concludes that this band probably does not consist of myosin but of some protein of similar solubility properties. By employing extraction procedures on myofibrils isolated from glycinerated fibres Hanson & Huxley (1953) have also obtained evidence that myosin is responsible for the *A* substance. These authors used the medium of Hasselbach & Schneider (1951) in the presence of 0·01 M-$MgCl_2$, or Guba-Straub solution (Guba & Straub, 1943) containing 0·0004 M-ATP, which solutions were considered to extract myosin only under the conditions employed, although the extracted protein was not tested for the presence of actin. After extraction with these solutions most of the *A* substance disappeared leaving small bands of dense material at the ends of the *H* space, and filaments could still clearly be seen running through the *A* band.

Both of these investigations indicate that there is a concentration of myosin in the *A* band and that probably this protein is the main component of the *A* substance. Neither studies allow us to say definitely, however, that myosin is not present in the *I* band, although results of electron-microscope studies of cross-sectioned muscle (Huxley, 1953) do suggest that the thicker filaments, which are considered to consist of myosin, are absent from this band.

If the myofibril is treated with salt solutions at lower ionic strength (less than 0·15), much less protein passes into solution. At ionic strengths of 0·10 and lower actomyosin does not pass into solution and although 0·15 M-KCl does not extract this complex in crude minces, extractants of similar ionic strength may have some solvent action on the myofibril if the state of comminution of the muscle is fine enough. For example, when fresh pigeon breast or rabbit muscle is homogenized in the Potter-Elvehjem homogenizer in 0·10 M-KCl containing 0·05-M-tris(hydroxymethyl) aminomethane buffer, pH 7·3, frequently the viscosity of the extract obtained after centrifuging off the myofibrils falls in the presence of ATP. Similar results have been obtained when this extractant contained in addition 0·001 M-ATP and 0·001 M-ethylenediaminetetraacetic acid.

On prolonged treatment with 0·078 M-borate buffer, pH 7·1, up to 13% of the total protein nitrogen of the myofibril passes into solution (borate-soluble fraction). About one-third of the protein in this extract is tropomyosin, representing 4% of the total protein in the myofibril, which is just the amount to be expected from the figures given by Bailey (1948) for whole muscle if all the tropomyosin is located in the myofibril and the latter is assumed to contain 65% of the total protein of muscle. Electrophoresis studies (Text-fig. 3) indicate that the protein other than tropomyosin in this

extract consists mainly of one component, which is as yet unidentified. The relative viscosity of the solution of the as yet unidentified protein is low, so far as can be judged from the properties of its mixed solution with tropomyosin, indicating that this new component does not appear to be unduly asymmetric in molecular form. This protein fraction represents 8–9% of the myofibril; it does not contain myosin, F-actin or G-actin or globulin X, and is not simply a contaminant of sarcoplasmic origin because the myofibrils have been washed free of sarcoplasm by repeated centrifugation during preparation. Evidence of complete removal of sarcoplasm is given by the fact that the purified myofibrils are free of creatine phosphokinase,



Text-fig. 3. Electrophoresis diagrams of the fraction obtained from rabbit myofibrils by prolonged extraction with 0·078 M-borate, pH 7·1. The fastest component is seen to be tropomyosin by comparing (*a*), which is the diagram obtained with the original extract, with (*b*) that obtained when pure tropomyosin is added to the extract. Composition of the buffer: 0·087 M-phosphate, pH 7·1; 0·25 M-NaCl (Perry, 1953).

and contain only slight traces of myokinase, both of which are typical sarcoplasmic enzymes.

Electron-microscope examination of such extracted myofibrils indicates that although there is evidence of filamentous structures, fine details cannot be recognized. This suggests that at least one of the components extracted by the 0·078 M-borate is of fairly general distribution in the filaments, and that its extraction partially destroys the filamentous structure. Another marked feature is the increased contrast of the outer edges of the *A* band compared with the interior of the band (see Pl. 1, fig. 2). Myofibrils isolated by the procedure described by Perry (1953) are partly contracted, and in consequence the *I* band is reduced in size. Hence when myofibrils prepared in this way are extracted with borate, the most frequent appearance is of

pairs of dense bands corresponding to the outer edges of two adjacent *A* bands separated by a narrower *I* band.

It has been suggested that tropomyosin may exist in the myofibril as a nucleic acid-tropomyosin complex (Hamoir, 1951). The evidence for this is that under certain conditions this protein can be crystallized from muscle extracts as a complex with nucleic acid, and in the case of carp muscle, for example, the nucleotropomyosin may contain as much as 15% nucleic acid. The total nucleic acid phosphorus content of the rabbit myofibril is about 0·04%, representing about 0·4% nucleic acid, which if it all were associated with the tropomyosin would give a nucleotropomyosin containing approximately 10% nucleic acid. The borate medium would not be expected to dissociate nucleotropomyosin as the ionic strength is too low, and consequently if it extracted tropomyosin at all it should extract the complex, if, indeed, the latter exists in the myofibril. This appears unlikely in rabbit myofibrils, for the nucleic acid present in the borate extract corresponds to a nucleotropomyosin containing 2·5–3·0% nucleic acid. This is estimated on the basis that all the nucleic acid is complexed with tropomyosin; probably the actual amount associated with this protein in the extract is much less.

This question of the nucleic acid content of the myofibril has some bearing on the work of Gerendas & Matoltsy (1948), who consider that the myofibril has a uniform positive birefringence which is compensated in the *I* band by the negative birefringence of a fibrous protein called the N-protein. Nucleic acids have negative birefringence, and from the rather meagre analytical details available the N-protein would appear to be a nucleoprotein. If all the nucleic acid in the myofibril was in fact concentrated in the *I* band the protein-nucleic acid ratio would still be about 30:1, and it seems unlikely that in this proportion the positive birefringence of the one could be compensated by the negative birefringence of the other. For this calculation it is assumed that the *I* band does not contain myosin (see Text-fig. 4 and accompanying text). If more protein is present in the *I* band than is represented in Text-fig. 4 the ratio is even less favourable. In view of this and the fact that so far no other worker has isolated the N-protein it appears that an explanation of the anisotropism of the *A* band is not provided by this substance if, indeed, such a protein exists. Neither tropomyosin nor the unidentified component of the borate-soluble fraction can be responsible, for after their extraction the birefringence of the *A* band so far as can be judged qualitatively is unimpaired.

It is of some interest to attempt a reconstruction of the myofibril in terms of the data which has already been presented. For this purpose the provisional analytical figures of Table 1 will be used to provide quantitative

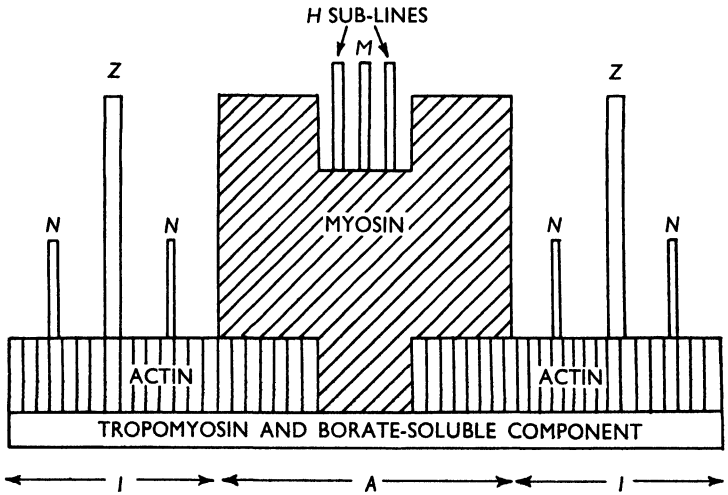
data for the model. In the first instance the following assumptions will be made:

(i) That all the myosin is localized in the *A* band (cf. Hanson & Huxley, 1953; Huxley & Hanson, 1954; and Hasselbach, 1953), the length of which is taken to be 1·4 times that of the *I* band.

(ii) That the actin filaments extend through the *I* band and through the *A* band excluding the *H* disk (Huxley, 1953) which is taken to be one-third the *A* band in width.

(iii) Tropomyosin and the unidentified component of the 0·078 M-borate extract are evenly distributed throughout the sarcomere.

(iv) Remaining 9% protein constitutes material of *Z*, *N*, *M* and *H* sub-bands.



Text-fig. 4. Diagram representing the localization of the protein components of the myofibril. The height of the shaded areas indicates the protein density at any point along the myofibril axis.

In Text-fig. 4 the sarcomere is represented in histogram form to indicate the total amount of protein in each band. The area of each shaded section represents the amount of a particular protein or group of proteins present, and as the isolated myofibril is of approximately equal width at the *I* and *A* bands the height of the shaded areas in the histogram represents the relative protein densities at any point along the axis of the sarcomere. It follows from the diagram that the average protein density of the *A* band is about three times that in the *I* band.

If the degree of hydration of the protein in all filaments is similar, they will be comparatively loosely spaced in the *I* band compared with those in the *A* band, with the result that the *Z* membrane will have an

important function as a spacer to prevent the I filaments aggregating laterally. A similar function in the *A* band has been suggested for the *M* line by the studies of Draper & Hodge (1949). Many workers have observed the structural weakness of the myofibril in the *I* band, particularly at the *Z* line and at the interface with the *A* band. Such observations and the contrast in the ability of the *I* bands to pick up electron and histological stains strongly suggest considerable disproportion in the amount of material present in these two bands. Certain implications of this disproportion in the distribution of the fibrous proteins between the *I* and *A* bands will be discussed in a later section.

ENZYMES ASSOCIATED WITH THE MYOFIBRIL

Classical histology has yielded little if any information on the enzymic function of the myofibril *in situ*. The information available has been derived from a study of myofibrils isolated from rabbit skeletal muscle by differential centrifugation of the homogenized tissue in media of comparatively low ionic strength. The procedure involves the removal of the soluble proteins and granular components of the sarcoplasm by washing the myofibrils in a large volume of salt solution of low ionic strength (Perry, 1952, 1953), and although the fine structure as seen with the electron microscope is well preserved during these procedures, it may well be that important soluble-enzyme systems closely integrated with the function of the myofibril *in situ*, yet representing only a very small fraction of the total myofibrillar protein, are lost during the preparation. The myofibrils contain about two-thirds of the total muscle protein, but when isolated they are comparatively inert enzymically in comparison with the mitochondria and soluble sarcoplasm which contain large numbers of enzymes concerned with oxidative processes and anaerobic metabolism respectively. In other words, within the protoplasm represented by the remaining one-third of the protein of the muscle cell are concentrated all the enzymes required for general cell function and the highly developed systems for supplying the energy requirement of the myofibril.

The characteristic enzyme of the myofibril is adenosinetriphosphatase (ATPase), closely associated with the myosin component, from which it has so far defied all attempts at separation. Purified myosin splits the terminal phosphate from ATP, but isolated myofibrils unless they are very carefully prepared to remove the last traces of myokinase will also slowly liberate phosphate from adenosinediphosphate (ADP) by the combined action of the latter enzyme and ATPase:

$$\text{ATP} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4, \qquad 2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{adenylic acid},$$

The myofibrillar ATPase will also split inosinetriphosphate, but chromatographic analysis indicates that very little of this substance is found in normal muscle extracts. By virtue of its close association with actin the myofibrillar ATPase shows some differences in properties to the myosin enzyme. For example, unlike myosin it is readily activated by Mg^{2+} (at ionic strength less than 0·15) and Mg^{2+} and Ca^{2+} do not have antagonistic effects on the myofibrillar ATPase. After treatment of the myofibril with M-KCl to bring most of the protein into solution, Mg^{2+} no longer activates the ATPase at ionic conditions under which the enzymic activity of intact untreated myofibrils is readily activated by this cation. On the other hand, the Ca^{2+} activation of the myofibrillar ATPase is unaffected by the M-KCl treatment. It now seems fairly clear that in the cell Mg^{2+} rather than Ca^{2+} is the ion concerned in activation of the contractile and ATP-splitting functions of the myofibril.

5-Adenylic deaminase has long been associated with purified myosin preparations, and likewise the isolated rabbit myofibril shows marked ability to deaminate 5-adenylic acid:

$$\text{Adenylic acid} \rightarrow \text{inosinic acid} + NH_3.$$

The majority of 5-adenylic deaminase in pigeon breast muscle is in the soluble sarcoplasmic fraction, but nevertheless in the rabbit the pattern of distribution does not appear so clear cut, for even after repeated washing myofibrils have Q_{NH_3} values for the deamination of adenylic acid of the same order as the Q_p values obtained with ATP as substrate. The 5-adenylic deaminase is not associated so tightly to the myosin as is the ATPase, for unlike the latter 5-adenylic deaminase slowly passes into solution on continued extraction of rabbit myofibrils with 0·078 M-borate, pH 7·1 (Hinds, 1954). It is of interest that Webster (1953) has recently reported the presence in a crude myofibrillar fraction from rabbit muscle of a further deaminating system which will deaminate ADP to form inosinediphosphate (IDP). This observation has been confirmed in the author's laboratory (Hinds & Perry, 1954) using purified myofibril preparations:

$$ADP \rightarrow IDP + NH_3.$$

The activity is comparatively low, in average preparations the rate of ADP deamination is 3–4% that obtained with adenylic acid at pH 6·1. Although the interpretation is somewhat complicated by traces of myokinase which are present, it seems that a true deamination of ADP is taking place in this system and that the IDP formation is not explained by the action of a phosphokinase of the type described by Krebs & Hems (1953), acting on traces of ITP in the ADP preparation:

$$ITP + ADP \rightleftharpoons ATP + IDP$$

Although it is generally considered that 5-adenylic deaminase is specific for 5-adenylic acid it has yet to be demonstrated whether a new enzyme or the myofibrillar 5-adenylic deaminase is responsible for the formation of inosinediphosphate (IDP) from ADP. Recently, Deutsch & Nilsson (1953) have reported actomyosin to have ADP deaminase activity.

As usually prepared (Perry, 1953, 1954*a*) myofibrils have slight myokinase activity, and it is difficult to remove the last traces of this enzyme even by repeated washing with solutions of low ionic strength. The myokinase can be removed at the expense of inducing contraction by treatment with ATP (Green, Brown & Mommaerts, 1953).

The close association of the myofibrillar ATPase with myokinase and 5-adenylic deaminase ensures that both labile phosphate groups can be split from ATP by the myofibril without the accumulation of adenylic acid. The sarcoplasm is rich in the two latter enzymes, and in the presence of sarcoplasmic extract the main product of ATP breakdown by myofibril preparations is inosinic acid.

The four enzymes described above are the only ones which have so far been demonstrated to be associated with the isolated rabbit myofibril. Clearly the ATPase is part of the structure of the myofibril, whereas the position of the remaining three is not so definite as they may have been adsorbed on to the myofibril from the surrounding sarcoplasm during isolation. In terms of contribution to the total protein of the isolated myofibril it is unlikely that enzymes mentioned above, other than the ATPase, account for more than 1 % of the whole. Certainly *in situ*, however, the considerable concentrations of myokinase and adenylic deaminase in the sarcoplasm will be in close contact with the myofibril.

Purified myofibril preparations have been shown to be free from the following enzymes: succinoxidase and pyruvic oxidase (Chappell & Perry, 1953), creatine phosphokinase (Perry, 1954*a*) and the glycolytic system (Perry, 1954*b*). It is clear from these results that the myofibril plays no part in the production of ATP; its role is primarily one of utilization, and the marked striation observed in skeletal and cardiac muscle is not specialization in the machinery of energy-rich phosphate production, but rather for its utilization and for intensified contractile function.

ADENOSINETRIPHOSPHATE AND THE MYOFIBRIL

The striking fact that ATP or the products of its dephosphorylation act as substrates for all the enzymes so far demonstrated to be present in the isolated myofibril emphasizes the importance of the role played by ATP in myofibrillar function. At the same time as it is broken down ATP induces

a change in form in the myofibril. With Mg^{2+} (0·005 M) and a medium of low ionic strength (0·025–0·20) the myofibril rapidly shortens in the presence of ATP, and the evidence both with the isolated system freed from sarcoplasm (Perry, 1954*a*), and with the myofibril still integrated into the cell matrix as in glycinerated fibre preparations (Weber & Portzehl, 1952), strongly suggests that this shortening is closely associated with the ATPase activity. In no instance so far has the shortening of the myofibril been demonstrated without the splitting of phosphate from ATP; on the contrary, shortening is most marked when the ATPase is working at optimum conditions.

In the muscle cell the myofibril is the main site of the utilization of chemical energy which has been converted into an available form by the glycolytic systems of the sarcoplasm and the oxidative systems of the mitochondria. The relative importance of these systems varies from muscle to muscle, and whereas skeletal muscle of the rabbit contains few mitochondria and is rich in anaerobic glycolytic systems for ATP production, the mitochondria are particularly numerous for this purpose in the muscles of high oxidative activity such as heart, pigeon breast and insect flight muscles. As the substrate for the enzymic and mechanical function of the myofibril ATP is the connecting link between these sites of energy metabolism, and one of the great problems of muscle function is to understand the mechanism of the control of this supply of chemical energy to the myofibril and its relation to the electrical stimulus which initiates contraction.

An approach to this problem is to investigate the distribution of ATP within the cell particularly with relation to the myofibril, but many difficulties beset such attempts due to the lability and ready diffusion of the nucleotide. Some information can be obtained by methods involving ultra-violet microphotometry of small areas of the living cell, but this technique, relying as it does on the characteristic absorption spectra of the purines, is unable to distinguish between adenylic acid, adenosine diphosphate and ATP. Also under such conditions these substances cannot be readily distinguished from nucleic acids and other nucleotides. Using this technique Caspersson & Thorell (1942) observed that the *I* band of resting muscle contained more material absorbing at 257 mμ than the *A* band, and concluded that in resting muscle there was a concentration of ATP in the former band. Hoagland (1946) made similar observations in that when muscle was photographed in ultra-violet light the *I* bands appeared dark in contrast to the *A* bands. Clearly there is more ultra-violet-absorbing material in the *I* band, but it is difficult to conclude finally from studies using the whole muscle that this absorption is actually due to the adenosine polyphosphates. Other possible causes of this difference in the ultra-violet

absorption of the two bands are (1) concentration of the myofibrillar nucleic acid (see Table 1) in the *I* band, (2) sub-microscopic granules of the microsome type, rich in adenine nucleotides or nucleic acids which may be arranged close to the *I* bands (I granules, cf. Heidenhain, 1911).

Isolated myofibrils analysed in bulk can be shown to contain small but definite amounts of adenine nucleotide which is almost entirely in the form of ADP (Perry, 1952). This nucleotide remains intact during storage of myofibril suspensions for several weeks at 0° C., and is not removed by repeated washings, but can be extracted by treating the myofibril with trichloroacetic acid. Chromatographic and enzymic analysis of trichloroacetic acid extracts of myofibrils clearly shows ADP to be the main nucleotide present. Rabbit skeletal muscle myofibrils contain on average about 110μg. of acid-labile P per g. dry weight, which represents 3·6μmole of ADP if all the labile phosphate exists as the latter substance. If the assumption is made that in resting muscle all the bound nucleotide is in the form of ATP, the myofibril would then contain about 10% of the total ATP in rabbit muscle. The ADP of the isolated myofibril is probably associated with the actin, for the amount of nucleotide present is in good agreement with the published figures for ADP content of purified actin if the latter protein is taken to be 23% of the total myofibril protein. The role of the ADP is not yet understood, for closely bound as it is to the structure of the myofibril it is in some way rendered unavailable to myokinase and 5-adenylic deaminase. These enzymes, working in conjunction with ATPase, rapidly convert most of the original ATP of the muscle homogenates, and any added ATP or ADP, into inosinic acid. This is readily demonstrated by following chromatographically the progress of ATP or ADP breakdown by purified myofibrils in the presence of a sarcoplasmic extract. If the sarcoplasmic protein in the incubation mixture amounts to 3% (in the cell it is probably 7–8 times as concentrated) inosinic acid is rapidly formed without any accumulation of adenylic acid. Nevertheless, in muscle homogenates the ADP bound to the myofibril is not broken down.

Wherever ATP has been found in biological systems enzymic mechanisms are usually present for its phosphorylation and dephosphorylation, and the implication is that similar systems are concerned with the metabolism of the bound nucleotide of the myofibril. Analogy might be drawn with the mitochondrion which also contains definite amounts of adenine nucleotide (Kielley & Kielley, 1951; Bartley & Davies, 1954). In the mitochondrion the nucleotide acts during oxidative phosphorylation as the built-in acceptor of energy-rich phosphate which then can be transferred to other acceptors or systems requiring it elsewhere in the cell.

The bound ADP would be an ideal acceptor for the energy-rich phosphate which is to be used during contraction, for built into the myofibril it would, when phosphorylated, provide ATP just at the site of its utilization and could be maintained in this form by the pressure of the phosphorylating systems of the sarcoplasm. If the myofibrillar nucleotide is maintained as ATP during a certain phase of the contraction cycle it is logical to assume that the ATPase of the myofibril is the dephosphorylating system. This is not, however, the only possible mechanism for the dephosphorylation of ATP, for it has been demonstrated that the polymerization of G-actin to F-actin involves the conversion of actin-bound ATP to ADP (Straub & Feuer, 1950). In such circumstances with ADP of the myofibril associated with the actin component, momentary changes in the state of phosphorylation of the nucleotide would be reflected by changes in the state of polymerization of the actin, the degree of polymerization being characteristic of the particular phase of the contraction cycle.

To date, however, there has been no evidence for *in vitro* phosphorylation of the myofibril-bound ADP. Unpublished experiments by the author (Perry, 1954*b*) failed to demonstrate any significant conversion of the myofibril-bound ADP to ATP during incubation with creatine phosphokinase and creatine phosphate under conditions favouring ATP formation. Shortening did not take place but could rapidly be induced by the addition of very low concentrations of free ADP to the system (Perry, 1954*a*). The amounts of added ADP were $\frac{1}{100}$ to $\frac{1}{10}$ the concentration of ATP required to bring about shortening in the absence of the creatine phosphokinase system. In this latter system creatine and phosphate were liberated in accordance with equations given below, and at the same time shortening took place:

$$\text{ADP} + \text{creatine phosphate} \rightleftharpoons \text{ATP} + \text{creatine},$$

$$\text{ATP} \rightarrow \text{ADP} + H_3PO_4$$

In the absence of added ADP neither creatine nor phosphate were liberated and the myofibril remained unchanged in length. Also when the low concentration of ADP which induced shortening in the presence of the creatine phosphokinase system is replaced by a similar concentration of ATP in the absence of the creatine phosphokinase system, little if any change took place. These investigations clearly show that contraction of the myofibril is induced not by a given low concentration of ATP but rather when there is a turnover of ATP produced in this case by the myofibrillar ATPase acting on catalytic amounts of ATP maintained by the creatine phosphokinase system. In other words, the extent of shortening depends on the rate of phosphate turnover rather than on the presence of a given concentration of ATP.

THE RELATION OF THE MYOFIBRILS TO THE SARCOPLASM

The myofibrils account for about 60 % to 65 % of the total protein in rabbit skeletal muscle, and if they occupy a similar percentage of the volume of the cell the average protein concentration will amount to roughly 20 %, although in the *A* band it will probably be much greater. The extra-myofibrillar space within the cell is occupied by the sarcoplasm and its various inclusions such as nuclei, and granules of various kinds including mitochondria. The number of the latter present depends very largely on the type of muscle and is particularly great in the highly active muscles, where they may occupy quite a high proportion of the extra-myofibrillar space. Ignoring for the moment the sarcoplasmic inclusions, the sarcoplasm is itself a concentrated protein sol, and if it simply occupied the extra-myofibrillar space its concentration would be of the same order as the average concentration in the myofibril. If, however, the myofibrils occupy a larger proportion of the cell volume than has been assumed above, the protein concentration of the sarcoplasm will be higher. Indeed, there is some evidence from the interference microscope studies on frog muscle by Huxley & Niedergerke (1954) that the protein concentration is higher in the sarcoplasm than in the *A* band. In general, the proteins of the sarcoplasm fall into the group which has globular rather than fibrous molecules, and concentrated solutions of sarcoplasm (made by dissolving freeze-dried sarcoplasm in water and containing up to 40 % protein) are of fairly low viscosity and will constitute the less viscous part of muscle cytoplasm. The sarcoplasmic proteins consist mainly of the enzymes of glycolysis and others such as creatine phosphokinase, myokinase, adenylic 5-deaminase, etc., whose molecules have diameters within the range 30–60 Å. The penetration of these molecules into the myofibrils must be seriously considered, particularly in the *I* band, where the filaments are quite widely spaced. For example, if most of the myosin is in the *A* band the protein density of the *I* band is such that the *I* filaments would be separated by spaces of about 250 Å. (Huxley & Hanson, 1954), whereas in the *A* band (excluding the H disk) the myofibril is more tightly packed with filaments, and penetration of the myofibril by protein molecules may be more restricted although electrolytes and such substances as ATP and creatine phosphate will be able to enter freely.

Penetration of the sarcoplasmic protein into the myofibril is strongly suggested in the white skeletal muscle of the rabbit which has few mitochondria and generally appears to rely on the anaerobic process of glycolysis to regenerate ATP. In this tissue the myofibrils within the muscle columns

closely associated and very little sarcoplasmic space is seen between em (Hodge, Huxley & Spiro, 1954). During contraction the myosin filaents are using ATP, the concentration of which is being maintained by zyme systems at the expense of creatine phosphate or glycogen breakwn, and it seems likely that this process is going on at the site of ATP lization, i.e. within the myofibril. Certainly if there is a relaxing factor esent in sarcoplasm to control the rate of ATP breakdown in muscle Iarsh, 1952; Bendall, 1953), this factor must be present in the interior of myofibril and close to the active centres of the myosin molecules.

This association of myofibrils and sarcoplasm must be a loose one, for ose sarcoplasmic proteins which penetrate into the myofibril would be pected to be readily washed out by repeated centrifugation in suitable dia under conditions in which the fine myofibrillar structure remains act. The enzymes myokinase and 5-adenylic deaminase, however, are ceptions in this respect, for the latter enzyme is not always removed by peated washing and it requires very persistent washing indeed to prepare yofibrils free from myokinase, facts which suggest that these enzymes in some way intimately associated with the proteins within the myofibril.

Any preferential penetration of sarcoplasm into the *I* band will lower figure for the ratio of protein densities in the *A* and *I* bands obtained measurement on intact muscle. Some information on this point could obtained by comparing the relative protein densities of the two bands in living fibre and in the isolated myofibril washed free from sarcoplasm. Recent work by Huxley & Niedergerke (1954) suggests that during isoic shortening only the *I* band alters in length. Similar conclusions have o been reached by Huxley & Hanson (1954) and both groups of workers ve suggested that during contraction the *I* band shortens by the filaments which it is composed (actin) moving into the *A* band between the *A* filaents (myosin). On the basis of this model any sarcoplasm which penetes the *I* band would be forced out and offer viscous resistance to the ortening. It also follows that some increase in width in the contracted iscle would arise from the sarcoplasm which has been forced out of the and.

Although the myofibrillar proteins are sharply contrasted with those of sarcoplasm so far as physical properties are concerned, in the cell the ictions of both types of protein are closely integrated. It seems unlikely it they exist as distinct entities separated by a definite interface such as mitochondrial membrane which acts as a barrier to the penetration of coplasm into the mitochondrion. In the *I* band of resting muscle at least ne penetration of the sarcoplasmic protein must take place. Apparently endowed with contractile properties in adult muscle the sarcoplasm and

its inclusions have the vital property of supplying energy-rich phosphate as ATP and regulating its utilization by the myofibril. This utilization of ATP by the myofibril involves complementary enzymic and contractile function, but the mechanism of the process is not yet understood. It seems clear that the key lies in the interaction of actin, myosin and ATP. Some progress has been made in relating these substances to the morphology of the myofibril, but real understanding of the process will not be achieved until the changes undergone by these substances at each stage of the contraction-relaxation cycle can be correlated with the fine structure of the myofibril.

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PLATE I

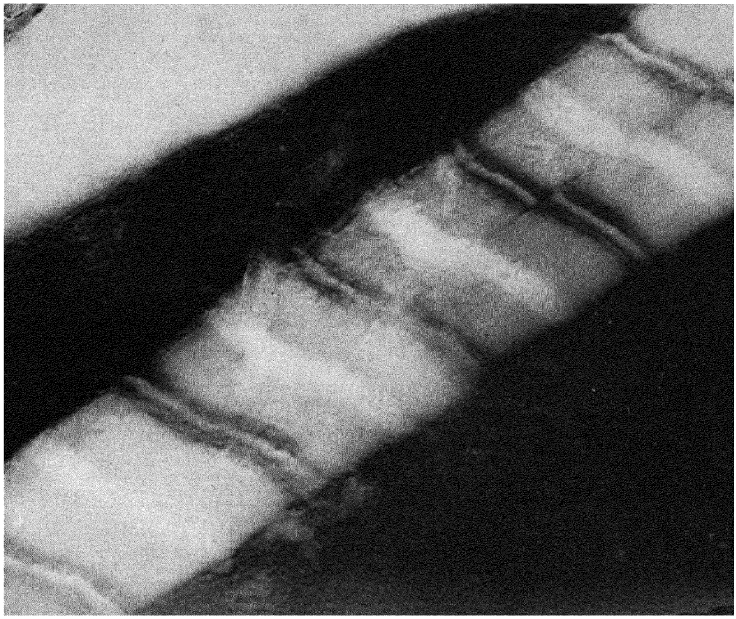

Fig. 1.

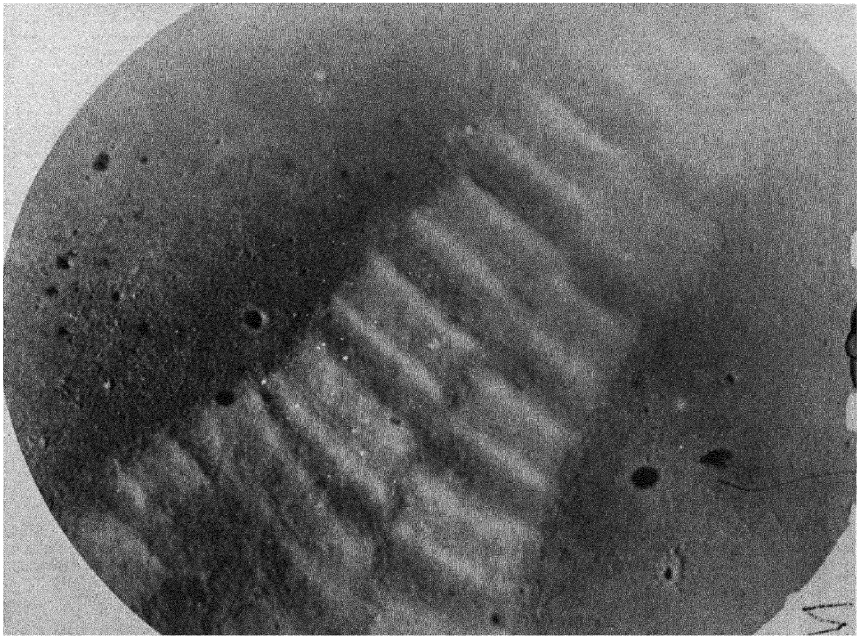

Fig. 2.

For explanation see p. 227

(*Facing p.* 226)

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EXPLANATION OF PLATE 1

Fig. 1. Electron micrograph of myofibrils from rabbit psoas muscle. Isolated by the collagenase method in 0·078 M-borate buffer, pH 7·1 (Perry & Horne, 1952). Gold palladium shadowed. × 18,000.

Fig. 2. Electron micrograph of rabbit myofibrils after extraction for 17 days at 4° C. in 0·078 M-borate buffer, pH 7·1. Prepared by mechanical disintegration of muscle in 0·078 M-borate buffer (Perry, 1953). Gold palladium shadowed. × 14,000.

THE STRUCTURAL BASIS OF CONTRACTION IN STRIATED MUSCLE

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I. INTRODUCTION

The behaviour of muscles presents one of the most interesting of all biological problems. The swift, powerful contraction of a striated muscle is a most dramatic manifestation of biological activity; and the problem of how such a contraction takes place can be approached by an exceptionally wide variety of techniques. In recent years, much attention has been given to the properties of the structural proteins that can be extracted from striated muscles. Two of them, myosin and actin, are of special importance. They may both be classified as fibrous proteins, although fibrous actin can be reversibly depolymerized to the globular form. Myosin is an enzyme for the dephosphorylation of adenosinetriphosphate (ATP); this reaction appears to be closely connected with the release of energy for contraction. Together, actin and myosin account for about half the dry weight of a muscle (Hasselbach & Schneider, 1951); and in myofibrils, which are the contractile elements of the muscle fibres, 80–90 % of the dry weight is made up of these two proteins (Perry, 1953). It is clear, therefore, that knowledge of the behaviour of actin and myosin must form the basis of any interpretation of muscular contraction in molecular terms. However, a living muscle and a purified protein are systems of greatly different complexities. It is very fortunate, therefore, that two other systems of intermediate degrees of complexity have been discovered and studied extensively by the schools of Szent-Györgyi (see Szent-Györgyi, 1951) and Weber (see Weber & Portzehl, 1952, 1954). These are the actomyosin thread and the glycerol-extracted muscle fibre.

* Some illustrations to this paper have already appeared elsewhere and we wish to acknowledge our thanks for permission from the respective editors to reproduce Figs. 3*a*, 4 and 7 from *Biochemica et Biophysica Acta*, Fig. 6 from the *Journal of Experimental Medicine*, Figs. 8 and 10 from the *Proceedings of the Royal Society*, B, and Figs. 19, 21, 24, 25, 26*b* and 32 from *Nature*.

The glycerol-extracted muscle fibre is a fibre that has been removed from a freshly killed animal, soaked in a mixture of equal volumes of water and glycerol at 0° C., and subsequently stored at −20° C.; rabbit psoas muscle is generally employed. The structural proteins remain undamaged and, by appropriate chemical treatments, the fibre can be made to contract and to relax again or can be made extensible. It cannot, however, be stimulated electrically. The extracted fibre may be regarded as a simplified fibre from which have been removed many of the factors that complicate the behaviour of the living system. The membrane is no longer excitable; the fibre is in a state of rigor; it has lost its ATP and, after thorough extraction, the ATP-restoring systems have been destroyed. It seems that the contractile structures have retained most of their special properties, whilst the other parts of the muscle have been rendered functionless.

The actomyosin thread is an even simpler system. When fresh muscles are minced and then stirred in a suitable hypertonic KCl solution, myosin and actin appear in the solution combined together as actomyosin; macroscopic threads of actomyosin relatively free from other proteins can be made from this solution by various methods. It is possible to make such threads contract and relax again, or to make them extensible. They behave like glycerol-extracted fibres, but they are weaker: the maximal tension exerted during contraction by an actomyosin thread is approximately 200–300 g./cm.^{2}, by a glycerol-extracted fibre about 4 kg./cm.^{2}, and by a fresh muscle preparation 4–5 kg./cm.^{2} (Weber & Portzehl, 1952).

Studies on these two simple systems, the glycerol-extracted fibre and the actomyosin thread, and studies on actin and myosin in solution, have led to a concept by which one can, to a limited extent, interpret muscular contraction in terms of the behaviour of myosin and actin. This concept is not quite universally accepted, and it is incomplete, but it affords a useful introduction to the results of the research we are to describe in this paper. The reviews of Mommaerts (1950, 1954), Szent-Györgyi (1951), Needham (1952) and Weber & Portzehl (1952, 1954) give accounts of the work on which this concept of muscle activity is based.

It is believed that the actin and myosin in an unstimulated living muscle fibre are prevented from forming actomyosin by the presence of ATP, which presumably occupies or masks the actin-binding sites on the myosin molecules. Such a fibre exhibits long-range elasticity; this property is attributed to the absence of actin-myosin linkages; hence ATP is said to have a 'plasticizing' effect under these conditions. When the fibre is stimulated, ATP is dephosphorylated to adenosine diphosphate under the influence of myosin, which is an ATPase; actin-myosin linkages are formed, and the fibre now exhibits normal, not long-range, elasticity. Moreover,

it shortens if it is free to do so, and it exerts tension. The energy for contraction is derived from the splitting off of the high-energy terminal phosphate group from the ATP. It is suggested that in the unstimulated fibre the ATPase activity of myosin is inhibited by a 'relaxing factor' (Marsh, 1952; Bendall, 1953); recent reports (Goodall & Andrew Szent-Györgyi, 1953; Lorand, 1953; Bendall, 1954) indicate that an essential part of the relaxing factor may be an ATP-restoring system. Stimulation of the contractile system is believed to consist in the inactivation of the relaxing factor. When stimulation ceases the relaxed state is restored by reactivation of the relaxing factor.

It has been the purpose of the present studies to reduce this somewhat abstract and incomplete biochemical picture of contraction to more concrete and precise structural terms. In these studies we have made extensive use of the glycerol-extracted *myofibril* of the rabbit's psoas muscle. These fibrils are about 1 μ in diameter, and are cross-striated (Fig. 1*). They are produced with great ease by fragmenting glycerol-extracted fibres in a Waring blendor. They lack both ATP and the relaxing factor. Contraction in this system is produced by giving ATP under conditions where myosin ATPase can function. Extensibility is produced by treatment with ATP under conditions where the activity of the enzyme is depressed.

The conclusions we have reached about the probable behaviour of actin and myosin during muscular activity are based on studies of the *structural* changes taking place in these cross-striated myofibrils during contraction or stretch (Huxley & Hanson, 1954). By what can only be considered as a piece of exceptional good fortune, the main striation of the fibril appears to be due to the localized distribution of myosin in one of the bands, the *A* band, whilst actin is apparently present both in the *A* bands and in the *I* bands that lie between them (Hanson & Huxley, 1953). These two proteins are the main constituents of the fibril, accounting for 80–90% of its dry weight (Perry, 1953). Hence, simple observations in the light microscope of the behaviour of the *A* and *I* bands during contraction and during stretch give immediate information about the behaviour of these principal protein constituents. This has been confirmed and extended by studies on stretched or shortened fibrils from which myosin has been extracted, actin being left behind. Much fuller understanding of the results of light microscopy has been gained from an examination in the electron microscope of very thin sections of fibrils fixed in various states; by this technique, high-resolution electron micrographs of the constituent protein filaments have been obtained (Huxley, 1953*a*). One of the chief difficulties in interpreting electron micrographs often lies in the evaluation of the artifacts produced

* Pls. 1–10 contain Figs. 1–33.

during preparation of the specimens. This difficulty has to some extent been avoided in our work, for low-angle X-ray diffraction studies have given more reliable, if less direct, information about some aspects of the submicroscopic organization of similar specimens in the unfixed wet state (Huxley, 1951, 1953*b*).

Previous ideas about the location of actin and myosin in muscle have followed rather a checkered course. Actomyosin was extracted and characterized for the first time by Kühne (see Kühne, 1868), who called it 'myosin', a name that was retained until Straub (1942) discovered actin and showed that earlier preparations of 'myosin' had contained the two proteins we now know as actin and myosin. As early as 1873 Engelmann reached the conclusion that 'myosin' was responsible for the birefringence of the *A* bands of muscle fibres. His conclusion later received strong support from the observation of Schipiloff & Danilewsky (1881) that most of the birefringence of muscles was destroyed when 'myosin' was extracted from them; they also found that dried drops of 'myosin' solutions were birefringent. Much later, von Muralt & Edsall (1930) and Noll & Weber (1934) demonstrated birefringence of flow in 'myosin' solutions and put on a sound basis the idea that 'myosin' was responsible for the birefringence of the *A* bands of muscle fibres. The first satisfactory electron micrographs of myofibrils (Hall, Jakus, & Schmitt, 1946) showed, however, longitudinal filaments extending through both *A* and *I* bands. Since these filaments remained straight in contracted fibrils, it was concluded that they were the elements that shortened. These results, taken in conjunction with the discovery that threads composed of actomysin were contractile (Szent-Györgyi, 1941), made it seem improbable that actomyosin was confined to the *A* bands. Various other suggestions were put forward to account for the large difference in birefringence between the *A* and *I* bands. Some evidence was found that negatively birefringent substances are localized in the *I* bands, where their presence might diminish the positive birefringence due to the myofilaments; these substances include phospholipins (Dempsey, Wislocki & Singer, 1946), nucleotides (Caspersson & Thorell, 1942; Engström, 1944; Hoagland, 1946; Pfeiffer, 1952) and the 'N-protein' of Banga & Szent-Györgyi (1940) (Matoltsy & Gerendás, 1948; Gerendás & Matoltsy, 1948*a*, *b*; Gerendás, Szarvas & Matoltsy, 1948; Matoltsy, 1949; Aloisi, Ascenzi & Bonetti, 1952). It was also widely believed that the high birefringence of the *A* bands could be accounted for by the presence of their characteristic '*A* substance', a material seen lying between the myofilaments.

In 1949 Rozsa, Szent-Györgyi & Wyckoff studied preparations of fibrous actin in the electron microscope and found a strong resemblance between

filaments of this protein and the myofilaments in myofibrils. This led Szent-Györgyi (1951) to the conclusion that myofilaments were composed of actin and that myosin was the *A* substance. (We understand that Szent-Györgyi no longer favours this view.) Amberson, Smith, Himmelfarb, Stout & Hoch (1950) briefly reported that the birefringence of the *A* bands could be destroyed by soaking muscles in pyrophosphate solutions which extracted, amongst other things, a protein identified as myosin. Ashley, Porter, Philpott & Hass (1951) also suggested that the '*A* substance' was probably myosin; but Snellman & Gelotte (1950) found that only the *I* bands were damaged when fibrils were subjected to myosin-extracting procedures and then examined in the electron microscope.

Hasselbach (1953) has recently carried out very carefully controlled extraction of myosin from muscles which he afterwards studied by electron microscopy; he has shown that myosin is indeed located in the *A* bands, and that longitudinal filaments, apparently composed of actin, remain in the fibrils after all the myosin has been extracted. Our own studies on the location of myosin (Hanson & Huxley, 1953) were carried out at the same time as those of Hasselbach, and we reached the same general conclusions simultaneously; but by employing the technique of ultra-thin sectioning for preparing our specimens, we were able to observe the details of the arrangement of the two proteins more precisely in the electron microscope. In particular, we found that myosin and actin are apparently confined to separate sets of filaments, the myosin filaments being present in the *A* bands alone, where they are the dominating feature, and the actin filaments extending through both *A* and *I* bands.

Previous work by light microscopy on the detailed changes in the cross-striations of muscles during contraction or stretch had given results which were unreliable, because the systems used either introduced artifacts of various kinds, or did not permit accurate measurement. When observations are made on intact muscle fibres in ordinary microscopes, optical artifacts can falsify the conclusions; the fibres are too thick for the high-resolution microscopy that is needed for observing and measuring the cross-striations, particularly while they are changing. We consider that this criticism applies to the work of Buchthal, Knappeis & Lindhard (1936, 1943) and to earlier work they review; it probably also accounts for the differences between our results and those of Horvath (1952), who used intact glycerol-extracted fibres. Histological sections can be used to overcome these difficulties, but results obtained on such material (see Jordan, 1920, 1933, and the earlier work he has reviewed) are still liable to be misleading because fixation artifacts and other uncontrolled distortions due to dehydration, sectioning, etc., are now present. Similar objections apply to some of the results of

electron microscopy (Philpott & Szent-Györgyi, 1953, and earlier work). In spite of these considerations, some of these previous papers gave results which can now be recognized as generally correct (for example, the studies of Speidel (1939) on living muscle fibres in the tails of tadpoles), although the details of the changes in band pattern were not known with the precision necessary for their interpretation. It is especially interesting that some of the most accurate observations ever made on fixed muscles were described by Engelmann in some of the earliest papers on this subject (Engelmann, 1873, 1878, 1880).

Optical artifacts such as those we have mentioned do not arise when one is using myofibrils 1–2μ in diameter; such fibrils are, in fact, very suitable objects for high resolution microscopy in phase-contrast illumination or in polarized light. As we have already mentioned, glycerol-extracted fibrils can be made to contract (Hanson, 1952) or be rendered extensible, by treatment with ATP under appropriate conditions. There is good reason to believe (see earlier discussion and references) that the structural changes taking place during their changes in length are the same as in living fibrils. This belief is confirmed by the results recently reported by A. F. Huxley & R. Niedergerke (1954), who have studied intact frog fibres by interference microscopy, another method which avoids artifacts. The changes they have observed in the relative lengths of the *I* and *A* bands of these fibres during stretch or during electrically stimulated contraction are the same as the changes we have seen during comparable behaviour of glycerol-extracted myofibrils. Similar changes have also been recorded (Harman, 1954) by cinéphotography of fibrils contracting and relaxing while in contact with active mitochondria.

A few details of some of the techniques we have employed are most conveniently inserted at this point. The glycerol-extracted fibrils have usually been equilibrated with a simple salt solution before observation or treatment. This will be referred to as 'standard salt solution'; its composition is: 0·1 M-KCl, 10^{-3} M-$MgCl_2$, 0·02 M-borate buffer (Palitzch), pH 7·0. Most of the observations by light microscopy have been made in a phase-contrast system employing an apochromatic objective of n.a. 1·15. Photographs were taken on fine-grain 35 mm. microfile film at a magnification of either 370 × or 550 ×. We estimate that our measurements of band lengths are accurate to within 5–10%. The techniques for electron microscopy have already been described by Hodge, Huxley and Spiro (1954*a*, *b*) and by Huxley (1953*a*).

II. THE STRUCTURE OF MYOFIBRILS AT REST LENGTH*

Figs. 1*a*, *b* are photographs taken (*a*) in a phase-contrast system and (*b*) between crossed polaroids, of a rest-length, glycerol-extracted myofibril from the psoas muscle of a rabbit. The fibril is in standard salt solution and it shows the following details characteristic of rest-length fibrils. At regular intervals along the fibril are *Z* lines, 2·3μ apart. The portion of the fibril between successive *Z* lines is known as a sarcomere. In the middle of the sarcomere is a long band (1·5μ in length) of high density (dark in phase contrast) and strong birefringence. This is the *A* band, so named because it is anisotropic in contrast to the *I* bands, which are almost completely isotropic. In the centre of the *A* band is a short zone of intermediate density (light in phase contrast) and weaker birefringence; this is the *H* zone. The *I* bands are the long zones (0·8μ) of low density in the phase-contrast photograph; they lie on either side of the *A* band and each of them is bisected by a *Z* line.

The *A* and *I* bands, the *Z* lines and the *H* zones are all easily recognizable in electron micrographs (Figs. 2, 4 and 6) of very thin longitudinal sections through rest-length, glycerol-extracted psoas fibres equilibrated with standard salt solution before fixation. In order to obtain sufficient contrast between the background and the thin filaments within the fibril the tissue has been 'stained' by fixation in osmium tetroxide and by subsequent treatment with phosphotungstic acid; the osmium and the tungsten both enhance electron scattering by the filaments. The *A* band in these sections (Fig. 2) possesses parallel filaments extending along its whole length; a second constituent, which sometimes appears filamentous, lies in between the 'primary' filaments of the *A* band, being absent, however, from the *H* zone. The filaments in the *I* bands are disorganized (probably as a result of fixation) but are clearly thinner than the primary filaments of the *A* band.

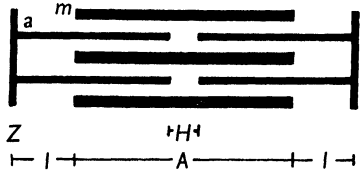
The arrangement of the filaments is better understood when accurately oriented transverse sections are examined (Figs. 3, 5 and 7) (Huxley, 1953*a*). The filaments are packed in a regular hexagonal array which extends continuously across each fibril. Fig. 3*a* is a section through the *A* band, cut to one side of the *H* zone. Fig. 3*b* is an enlargement of one part of this section, and it shows that there are two kinds of filaments, lying in a two-fold hexagonal array. Around each of the thicker filaments (about 110Å. in diameter) are six thinner filaments (about 40Å. in diameter) which it

* Rest length is the length assumed by the intact psoas muscle in the body when a completely relaxed rabbit (e.g. one killed by myanesin treatment) is laid on its back.

shares with its six nearest neighbours; thus each thinner filament lies equidistant from three thicker filaments. The distance from centre to centre of adjacent thicker filaments is between 200 and 300 Å. In the *H* zone (Fig. 5) the thinner filaments are absent, and the thicker filaments are frequently of greater diameter (about 140 Å.) than in the rest of the *A* band. In transverse sections through the *I* bands the filaments are irregularly arranged and are often clumped together, probably because they are difficult to fix and prepare for sectioning. The diameter of an *I* band filament is approximately 40 Å. Fig. 7 is a transverse section across a number of fibrils and intervening sarcoplasm (mitochondria are visible) in a frog's sartorius muscle, held at rest length and fixed without prior glycerol-extraction; sections through *A* bands and *H* zones are recognizable by their characteristic filaments. In part of Fig. 4, which is a longitudinal section through glycerol-extracted rabbit psoas fibrils, the microtome knife has chanced to pass through an array of the thinner filaments in one *A* band (indicated by an arrow) and apparently slightly below a layer of *H* zone filaments in this sarcomere. We believe that these thin filaments in the *A* band are continuous with, and identical to, the thin filaments in the *I* band.

The procedures used in preparing these thin sections have undoubtedly distorted to some extent many of the structural details of the fibrils; it is dangerous to assume that the appearance of specimens fixed and stained in this way necessarily gives a true picture of the arrangement of protein in the living or the glycerol-extracted muscle. It is important, therefore, to make use of independent methods to investigate this arrangement. One of us (Huxley, 1951, 1953*b*) has obtained low-angle X-ray diffraction photographs of unfixed, rest-length, glycerol-extracted rabbit psoas muscle fibres equilibrated with standard salt solution and examined while in this solution (Fig. 8) and also of living frog sartorius muscle (Fig. 10). Before electron-microscope studies had been undertaken, the X-ray diffraction data on the glycerol-extracted muscle had been interpreted as indicating the presence in the fibril of a two-fold hexagonal array of longitudinal filaments (Fig. 9). The distance from centre to centre of filaments in the primary array was found to be 455 Å. In electron micrographs this distance is 200–300 Å.; thus some lateral shrinkage has taken place in the fibril during specimen preparation. It is not known by how much the diameters of the filaments themselves have been diminished by dehydration or increased by electron staining. Other X-ray diffraction photographs obtained from the same type of preparation show that there is also an axial periodicity of 415 Å.; this is presumably represented in electron micrographs by the fine cross-striations, present in both *A* and *I* bands, that are sometimes visible in longitudinal sections (Fig. 6).

Glycerol-extracted fibres are in a state of rigor; it is interesting, therefore, to compare their submicroscopic organization with that of extensible fibres, either fresh relaxed fibres, or glycerol-extracted fibres plasticized by treatment with pyrophosphate (see Weber & Portzehl, 1954). X-ray diffraction photographs of extensible fibres (Fig. 10 is of a surviving frog sartorius preparation in Ringer solution) show that the primary hexagonal array of filaments is present, but suggest that the material between them is randomly arranged (Fig. 11). When frog fibres have been exhausted by repetitive stimulation in the presence of iodoacetate (a method of depleting their ATP content), the diffraction pattern they give is now the same as that of glycerol-extracted muscle, i.e. the material between the primary filaments is now regularly arranged. It was suggested (Huxley, 1953*b*) that the two



Text-fig. 1. Diagram of the arrangement of filaments in one sarcomere of a myofibril at rest-length; *a*, actin filament; *m*, myosin filament.

arrays correspond to actin and myosin filaments and that the existence of myosin-actin linkages in the absence of ATP stabilizes the secondary array of filaments in the interstices of the primary array, thus enabling them to be detected by X-ray diffraction methods. (It now seems likely that the attachment of actin to myosin in glycerol-extracted muscle is also responsible for the greater ease with which the secondary filaments can be fixed in the *A* bands of such preparations for electron microscope study.) In the presence of ATP, actin-myosin linkages are thought to be absent, and it was suggested that under these conditions the secondary filaments are randomly arranged and therefore do not reveal themselves in X-ray diffraction photographs. It was not known, however, at the time of the X-ray studies, that the primary filaments were confined to the *A* bands, nor was it certain which array corresponded to which protein.

This account of the structure of rest-length fibrils can now be summarized in a diagram (Text-fig. 1) to which other details will later be added (Text-figs. 3–8).

III. LOCATION OF STRUCTURAL PROTEINS IN MYOFIBRILS AT REST-LENGTH

Information about the composition of myofibrils is still very incomplete, but it is clear that structural proteins account for about 90% of their dry weight. The data of Hasselbach & Schneider (1951) and of Perry (1953) suggest that most of this material consists of actin and myosin, and that there is about three times as much myosin as actin. The quantity of tropomyosin in the fibril is about 4% of its dry weight (Perry, 1953). Little is known of other protein constituents, the presence of which is suggested by observations of Perry (1953), Dubuisson (1950) and Green, Brown & Mommaerts (1953). Since actin and myosin are the chief constituents of a glycerol-extracted fibril, from which virtually all but the structural proteins have been removed, it appears likely that the myofilaments revealed by electron microscopy are composed almost entirely of actin and myosin. It is natural to suggest that the two types of filaments may differ in their composition, one containing myosin and the other actin. Calculations of the relative amounts of material in the two types of filaments have been made (see Huxley, 1953*a*); they are based on measurements of filaments in electron micrographs, and it is assumed that the filaments are solid. The ratio of the weight of material in the filaments in the primary array to the weight of material in the filaments in the secondary array is calculated to be 2·7:1. The concept that the former consist of myosin and the latter of actin is in reasonably good agreement with the finding that the ratio of the weight of myosin to the weight of actin in muscles is approximately 3:1 (Hasselbach & Schneider, 1951).

Much more reliable information about the location of myosin and actin in myofibrils has been obtained by observations on their structure after they have been treated by methods designed to extract all the myosin from them. The extracted fibrils remain intact, but show marked structural changes. They also lose their ability to contract when treated with ATP.

The classical method for dissolving myosin from rabbit muscles (see Szent-Györgyi, 1951) is as follows. Fresh muscle, cooled to 0° C., is minced and then stirred for about 15 min. in Guba-Straub solution (0·3M-KCl, 0·15M-phosphate buffer, pH 6·5). Fairly pure myosin goes into solution, but the yield is small; with longer extraction actomyosin appears in the solution. Hasselbach & Schneider (1951) have described a different method, claimed to extract all the myosin in the muscle and give a solution uncontaminated with actin, for there is no change in viscosity on addition of ATP (pyrophosphate having been removed by dialysis). In this procedure fresh minced muscle is stirred for several hours at 0° C. in a

solution of 0·47M-KCl, 0·01M-pyrophosphate, 0·1M-phosphate buffer, pH 6·4; after long extraction no more protein goes into solution. From the residue actin can be extracted, apparently uncontaminated with myosin.

We have examined, by phase contrast and polarized light microscopy, minced muscle residue sampled at various times during extraction of myosin by Guba-Straub solution or by Hasselbach & Schneider's pyrophosphate solution (abbreviated to 'H-S solution'). It was not feasible to break up the residue in order to obtain fragments suitable for high-resolution microscopy, because such a procedure effects further protein extraction. Therefore samples were fixed in formaldehyde or osmium tetroxide and, since they did not cleave into fibrils on blending, frozen sections or paraffin-imbedded sections were prepared. Studies on this material showed that during the course of myosin extraction the overall density and the birefringence of the *A* bands greatly diminished (Figs. 12–14). A narrow zone in the centre of the *A* band retained a high density during the early stages of extraction in H-S solution (Fig. 13); in the later stages the fibre structure became greatly disorganized, but some stripes were still recognizable (Fig. 14).

These methods for studying structural changes during myosin extraction were unsatisfactory in several respects, and an important improvement was made by using isolated myofibrils instead of minced muscle, and observing the fibrils in the microscope while they were being treated with myosin-extracting solutions. In this way, the whole course of the structural changes has been followed, and the identity of the bands in the extracted fibrils accurately determined. Although most of the observations have been made on glycerol-extracted fibrils, the results have been checked by using fibrils from untreated muscle blended in standard salt solution, or fibrils prepared by the method of Schick & Hass (1950) which employs weak trypsin solutions to help in dissociating the fibres. Since all these fibrils were in rigor, having lost their ATP, the Guba-Straub solution had to be modified by adding an agent, in this case 4×10^{-4}M-ATP, in order to dissociate myosin from actin. H-S solution includes pyrophosphate which presumably acts in the same way, but it was found necessary to include some magnesium (about 10^{-3}M-$MgCl_2$). Treatment of fibrils with either of these solutions produces swelling which can be reversed by treatment with standard salt solution. This swelling is independent of the significant structural changes we shall describe: thus, Guba-Straub solution without ATP, or H-S solution without magnesium, bring about reversible swelling without any other visible structural change.

Brief treatment of a rest-length glycerol-extracted fibril with either Guba-Straub solution + ATP or H-S solution + $MgCl_2$ causes a rapid and

irreversible decrease in the density (Figs. 15, 16) and the birefringence of the *A* bands. The fibril that is left has a 'backbone' of approximately the same density as that of the original *I* bands, and a new pattern of cross-striations; these are the *Z* lines and a dark line in the centre of each sarcomere. This dark line disappears if the treatment with H-S solution is prolonged. Hasselbach (1953), also, considers that this line, which he found in electron micrographs, contains unextracted myosin. We have found that it can be rapidly removed by a pyrophosphate solution of high ionic strength (0·1 M-sodium pyrophosphate, 0·01 M-phosphate buffer, 10^{-3}M-$MgCl_2$, pH 7·0). This solution, applied to intact fibrils, rapidly removes all the dense material of the *A* bands (Figs. 17 and 18*a*, *b*), their high birefringence disappearing at the same time.

A rest-length fibril from which all the myosin is believed to have been extracted has the following structure (Figs. 17*b*, 24). The *Z* lines are intact, and material of very low birefringence and low density, approximately that of the original *I* bands, extends from the *Z* lines towards the centre of the sarcomere, where it terminates at the boundaries of a zone that appears to be a gap in the fibril structure. This region of virtually zero optical density is apparently the same length as the *H* zone of the intact fibril. Although no material is visible in this zone in extracted fibrils, some structures must cross it, because such fibrils can be stretched (by a micro-manipulative technique described on p. 241) and will shorten when released; and they do not break up even when lightly centrifuged. During stretch it is the 'gap' that elongates.

These results, obtained by using established procedures for extracting myosin (or justifiable modifications of these methods), lead to the conclusion that myosin is located in the *A* bands and is responsible for their high density and birefringence. More precise localization of myosin in the fibril has been achieved by examining in the electron microscope thin sections of glycerol-extracted fibres treated with H-S solution or with 0·1 M-pyrophosphate solution (Figs. 19 and 20). In incompletely extracted fibrils (Fig. 19) parts of the filaments of the primary array are still present in the centre of the sarcomere. In the most completely extracted material (Fig. 20) the primary array of thicker filaments (110 Å. in diameter) has entirely disappeared from the whole of the *A* band. The thinner filaments (about 40 Å.) are still present, and it can be seen that they extend from the *Z* lines to the borders of an empty zone in the centre of the sarcomere; we presume that we have not yet succeeded in preserving the material undoubtedly originally present in this zone.

We conclude that the myosin of the myofibril is located in the filaments of the primary hexagonal array, which are confined to the *A* bands and

extend along their whole length. When these filaments have been removed, at least three other components are left behind: the thin filaments of the secondary hexagonal array, the material of unknown structure present in the central zone of the sarcomere, and the *Z* lines. It is probable that the *Z* line material is accounted for in the 'stroma' protein fraction of whole muscle. Actin, tropomyosin and any other structural proteins of the fibril are therefore presumably located in the thin filaments and in the material in the centre of the sarcomere. It is thought that the fibril contains about five times as much actin as tropomyosin. (About 13% of the total muscle protein is actin (Hasselbach & Schneider, 1951) and 2·6% is tropomyosin (Bailey, 1948).) Thus it appears likely that the thin filaments, which are the most important constituents of the sarcomere after myosin has been removed, consist predominantly if not entirely of actin. This conclusion is supported by the preliminary results of observations on fibrils during treatment by either the method of Tsao & Bailey (1953) or the method of Andrew Szent-Györgyi (1951) for actin extraction (Fig. 18). After prior myosin extraction, the residual material of the sarcomere between the *Z* lines and the edges of the *H* zone is apparently removed by actin extraction, for the optical density in this region falls to virtually zero. The *Z* lines, however, are not removed, and each of them often becomes a double line.

We therefore believe that in the rest-length myofibril myosin and actin are located in different filaments; the myosin filaments are confined to the *A* bands, whilst the actin filaments extend from the *Z* lines to the boundaries of the *H* zone in the centre of the *A* band (see Text-fig. 1). We also think that the two sets of actin filaments in the sarcomere are joined to each other in the *H* zone by elements of unknown structure, which we are provisionally calling the '*S* filaments' because they can stretch (see § V). It is likely that the myosin filaments in the *H* zone are cross-linked to the *S* filaments; possibly these links differ from the myosin-actin links in the rest of the *A* band, and so account for the observed differences in extractibility of myosin from the two different regions of the *A* band.

IV. STRUCTURAL CHANGES DURING CONTRACTION

(*a*) *Changes in the cross-striation*

When rest-length glycerol-extracted fibrils in standard salt solution are treated with ATP (about 5×10^{-4}M) they will contract down to about 30% rest length, and some of the structural changes taking place during shortening can be followed in the light microscope (Hanson, 1952). This type of contraction, in which the fibrils are shortening under virtually zero

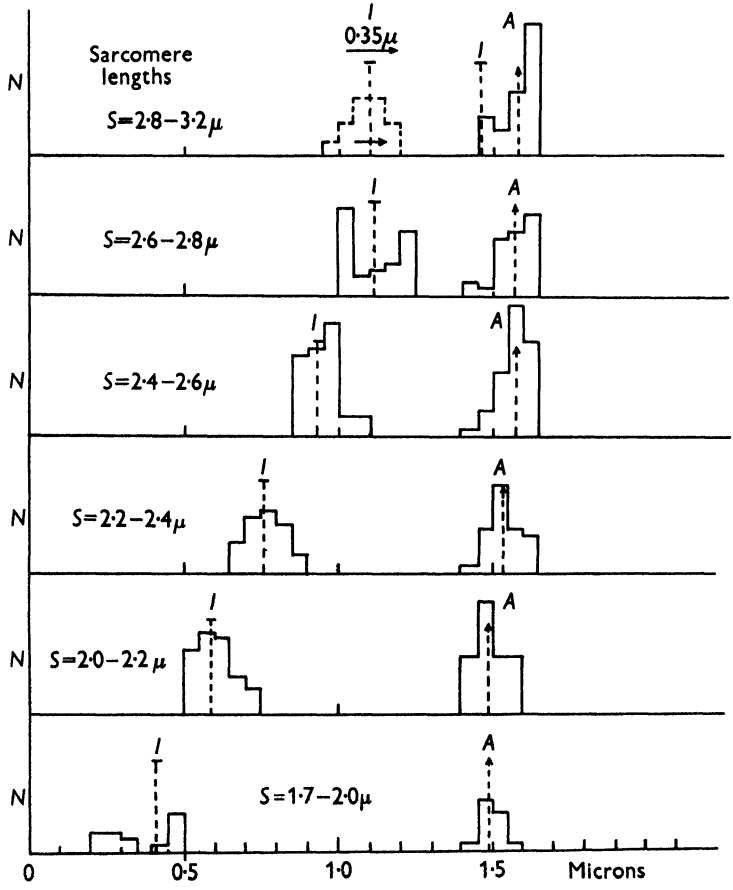
load, has been studied further (Huxley & Hanson, 1954) by cinéphotography and, more conveniently, by visual observation of systems in which either the speed of shortening was decreased, or in which shortening proceeded in steps. The same results were obtained in all cases. Slow contractions were produced when the fibrils were treated with ATP in a cold room (2° C.) and the concentration of magnesium ions kept at a very low level; it is known that under these conditions the rate of dephosphorylation of ATP is depressed (Weber & Portzehl, 1954). It was found possible to produce step-wise contraction in fibrils at room temperature (about 22° C.) by controlling the amount of ATP that reached them. The fibril preparation under the microscope was irrigated with a small drop of very dilute ATP (about 5×10^{-6}M) in standard salt solution, and as soon as shortening was noticed irrigation was stopped. By successive treatments with drops of dilute ATP, contraction down to about 30% rest length could be achieved in a series of short steps, and the structural changes observed at each new length recorded on fine-grain film so that accurate measurements of *A* and *I* band lengths could be made.

Observations on fibrils contracting isometrically were made possible by a simple micromanipulative technique. A thin preparation of fibrils mixed with fibre fragments was searched until a fibril was found with one end imbedded in a fragment adhering to the cover-slip and its other end held in a fragment attached to the slide. If firmly held, such a fibril will not shorten when treated with ATP, but controlled shortening can be obtained by moving the coverslip in the appropriate direction. Very small movements can be made with great ease, for the thin layer of liquid in the preparation provides smooth and highly viscous lubrication.

Fig. 21 illustrates the structural changes observed during contraction under virtually zero load. The most important event is the shortening of the *I* bands which disappear at about 65% rest length; meanwhile the length of the *A* bands remains virtually constant at $1{\cdot}5\mu$. When the *A* bands have been brought into contact with the *Z* lines (65% rest length), further shortening involves the formation of contraction bands around the *Z* lines, then thickening of the contraction bands and further decrease in the length of the sarcomere.

While the *I* bands are shortening, changes in density take place within the *A* bands, although these bands remain at virtually constant length (Figs. 21, 22 and 27). The *H* zone, of low density in the rest-length fibril, is no longer distinguishable from the rest of the *A* band when the fibril has shortened to about 90% rest length. At about 80% rest length three lines of exceptionally high density have appeared in the *A* band, one in the centre and one at either end. The two terminal lines contribute to the

contraction bands, which form where the *A* bands come into contact with the *Z* lines. Meanwhile, the dense line in the centre of the sarcomere is replaced by two lines (Fig. 23) which are eventually incorporated in the contraction bands at about 30% rest length.



Text-fig. 2. Results of measuring sarcomere lengths (*S*) and *A* and *I* band lengths in photographs of glycerol-extracted rabbit psoas fibrils. Fibrils with *S* of more than 2·4μ were obtained from fibres held stretched during glycerol-extraction. Those with *S* of less than 2·2μ had been treated with ATP, and the measurements were made on *series* of photographs taken at stages during step-wise shortening. For the purpose of expressing the results, fibrils of similar sarcomere lengths have been grouped together, and the ordinates (*N*) represent the number of fibrils measured in each group. The average *A* or *I* band length in each group is indicated by a vertical dotted arrow.

Isometric contraction involves no change in the lengths of either the *I* bands or the *A* bands; but within the *A* band we have observed changes in density similar to those taking place during the early stages of free shortening: the *H* zone disappears and is replaced by a very dense line. If

the fibril adheres to the cover-slip or slide along its whole length, then no changes take place within the *A* bands of the immobilized sarcomeres.

Our belief that the structural changes taking place during ATP-induced contraction of glycerol-extracted fibrils are exactly similar to those occurring during the contraction *in vivo* has been confirmed by the recent work of A. F. Huxley & R. Niedergerke (1954), who have shown that the *I* bands shorten in surviving frog fibres during a single twitch or a short tetanus, whilst there is virtually no change in the length of the *A* bands; they have also reported changes of density within the *A* bands. It is also apparent in the film made by Harman (1954) of contraction of myofibrils in contact with active mitochondria that the *I* bands shorten and the *A* bands show no obvious changes in length.

A further control for our experiments was provided by studying the cross-striations of fibrils with short sarcomeres, which are often found in preparations of glycerol-extracted fibrils. Presumably some of the fibres of the psoas muscle are stimulated and contract while they are being prepared for glycerol extraction. It is found that the *A* band length is virtually constant (see next paragraph), whilst the *I* band length and the pattern of lines within the *A* band change with sarcomere length in the manner we have observed during ATP-induced contraction.

In Text-fig. 2 are shown the results obtained by measuring *A* and *I* band lengths in photographs taken during ATP-induced step-wise contraction of many different fibrils. Some of the histograms in this figure refer to stretched fibrils (sarcomere lengths of more than 2·4μ) described on p. 246. Although our measurements apparently demonstrate a slight increase in *A* band length during stretch and a slight decrease during contraction, the resolution of the light microscope is inadequate to establish the reality of these changes in *A* band length (only 0·1μ over the whole range from 75% to 140% rest length). The figure represents the results of several hundred different measurements of sarcomere lengths.

(*b*) *Behaviour of the structural proteins*

By the methods already described, myosin has been extracted from fibrils with sarcomere lengths ranging from rest length down to about 30% rest length. In Pl. 7 are shown photographs of extracted fibrils together with untreated fibrils of similar sarcomere lengths. It can be seen that the changes in density that take place within the *A* bands during shortening are apparently due to constituents other than myosin. Thus, when the *H* zone has disappeared at about 90% rest length its place is taken by material of the same optical density as occupies the rest of the myosin-free sarcomere (Fig. 25). This material now fills the site of the

'*S* filaments'. At 85% rest length or less, the three dense lines in the *A* band are found to belong to the material that is left after myosin extraction (Fig. 26). Similarly, in excessively shortened fibrils, part of the contraction band material remains after the myosin has been removed.

Thin sections through fibres that were treated with ATP and allowed to shorten before fixation have been studied in the electron microscope (Pl. 9). The technique of specimen preparation probably causes uncontrolled changes in *A* and *I* band lengths, due both to fixation artifacts and to section compression, but it is permissible to conclude that the length of the *I* bands indeed diminishes during shortening of the muscle fibres, and that eventually the *I* band filaments are completely retracted into the *A* bands. It is found that during the early stages of shortening thin filaments of the secondary array appear in the centre of the *A* band, so that an *H* zone is no longer distinguishable. In sarcomeres that had shortened further, dense lines are found in the *A* band, corresponding to those observed by light microscopy. After contraction bands have formed, the thick filaments between them remain straight. Preliminary observations on contracted fibrils from which the primary array of thick filaments has been removed by myosin-extracting solutions, confirm the conclusion that contraction involves translation into the *A* bands of the thin filaments of the secondary array. They first of all move into the '*S* filament' zone in the centre of the sarcomere, and later form dense bands in this region and at either end of the *A* band. In excessively shortened sarcomeres they form part of the contraction bands.

Therefore we can now conclude that contraction down to about 65% rest length involves translation of actin filaments into the array of myosin filaments in the *A* bands, and does not involve any significant changes in the length of the filaments composed of myosin (*A* band length remains virtually constant). The actin filaments first of all remain at apparently constant length as they slide in and fill up the *H* zone in the centre of the sarcomere (contraction down to about 90% rest length); when the fibril contracts below this length the actin filaments become shorter, but whether this is a cause or an effect of contraction, we cannot tell. Some shortening may take place along the whole length of the actin filaments, but it appears that in the centre of the *A* band and at its two ends the actin filaments fold up or coil in a more pronounced manner. Below 65% rest length, both myosin filaments and actin filaments fold up in the contraction bands, but the myosin filaments remain straight in the rest of the sarcomere.

(*c*) *Position of the anisotropic band in contracted fibrils*

Although many careful observations have been made on the birefringence of myofibrils, it does not yet seem to be sufficiently firmly established that there is no change in the relative *positions* of the anisotropic and isotropic bands within the sarcomere during contraction (see reviews of the literature in Jordan, 1920 and Schmidt, 1937). Like Bennett & Porter (1953) we have confirmed the conclusions of Engelmann, Rollett and other early investigators that in muscles which have shortened until contraction bands have formed those contraction bands are isotropic, and the rest of the sarcomere is anisotropic. Our observations have been made not only on rabbit fibrils but also on the fibrils of the indirect flight muscles of a number of insects where the sarcomeres are longer and easier to study in polarized light. Fibrils taken from freshly killed insects (a large number of species from Neuroptera, Ephemeroptera, Hemiptera, Coleoptera, Lepidoptera, Hymenoptera, Diptera) nearly always have contraction bands. Fig. 29 shows a fibril of the blow-fly *Calliphora erythrocephala* photographed (*a*) in a phase contrast system and (*b*) in polarized light; Fig. 28 is a similar pair of photographs of a contracted rabbit fibril (compare Fig. 1 showing a rest-length fibril). During contraction, the boundaries of the anisotropic bands remain the same as those of the denser bands, and when adjacent denser bands come into contact at the *Z* lines, the region between the *Z* lines is anisotropic, although immediately around the *Z* lines, where contraction bands have formed, the fibril is isotropic. These observations bear out the conclusions we have reached about the behaviour of the myosin filaments during contraction; and the isotropic appearance of the contraction bands also supports our conclusion that they are formed from filaments that crumple up at the ends of the sarcomeres.

V. STRUCTURAL CHANGES DURING STRETCH

(*a*) *Changes in the cross-striation*

Isolated myofibrils have been stretched, while under observation in the light microscope, by the simple technique already described. Two types of extensibility have been studied, and we consider that in the one case the state of the fibril corresponded to that of a relaxed living muscle fibre, whilst in the other case we were probably observing the same changes as occur during stretch of a muscle in a state of rigor or during contraction, i.e. stretch of the series elastic component (Hill, 1950).

In order to observe the first type of stretch, fibrils were kept at about 2° C., were washed in magnesium-free standard salt solution, and were

treated with a high concentration of ATP (about 10^{-2}M). Under these conditions the ATP is an efficient plasticizer; myosin ATPase activity is depressed (Weber & Portzehl, 1954). Extension of these fibrils brings about increase in the length of the *I* bands; the length of the *A* bands remains constant at $1{\cdot}5\mu$. While the *I* band lengthens, the *H* zone in the centre of the *A* band becomes correspondingly longer. This process is reversible; when the fibrils are released they will shorten until contraction bands are formed, and they can be re-extended.

Other fibrils were stretched in the presence of 10^{-4}M-ATP and 10^{-3}M-$MgCl_2$ at room temperature (about 22° C.). Under these conditions the fibrils are exerting a strong tension. Similar results were also obtained by stretching fibrils in rigor (no ATP in the standard salt solution). In each case the *I* bands lengthened and the *A* bands showed no change in length. In neither case, however, did the *H* zone elongate. A change in the *Z* lines was noticed in these fibrils; they became blurred, which suggests that the site of the extensible component may be at the *Z* lines. No changes in the *Z* lines were found during stretch of plasticized fibrils. Both types of stretch are reversible.

Our belief that the changes we observe during stretch of plasticized fibrils correspond to the changes taking place during stretch of unstimulated muscles *in vivo* has been strengthened by the results reported by A. F. Huxley & R. Niedergerke (1954) who found, by interference microscopy, that extension of unstimulated surviving frog fibres is accomplished by extension of the *I* bands alone, no resolvable changes taking place in the length of the *A* bands. Elongation of *I* bands and *H* zones in fibrils that are being stretched is also noticeable in the film made by Harman (1954) of the reversible contractions he obtained in systems of fibrils and active mitochondria. Furthermore, we have made numerous measurements of *A* and *I* band lengths in glycerol-extracted fibrils obtained from rabbit psoas fibres that were stretched immediately after removal from the animal, before glycerol-extraction (Text-fig. 2). We have found that the length of the *I* band is variable, and nearly always in excess of $0{\cdot}8\mu$, its length in a rest-length fibril; the length of the *A* band is virtually constant (see p. 244). In fibrils with long *I* bands, the *H* zone is correspondingly elongated (Fig. 32).

(*b*) *Behaviour of the structural proteins*

In electron micrographs of intact stretched fibrils cut in longitudinal section (Fig. 31), it is clear that the *H* zone is elongated, i.e. that the central region of the *A* band, distinguished by the absence of the thinner filaments of the secondary hexagonal array, occupies a proportionately greater part of that band.

Myosin has been extracted from stretched fibrils obtained from muscles that were extended before glycerol treatment. The most noticeable feature in such an extracted fibril in the phase-contrast microscope is the long zone of virtually zero density in the centre of the sarcomere (Fig. 32*b*). This zone corresponds in length to the elongated *H* zone of the intact fibril (Fig. 32*a*). In electron micrographs of thin sections through fibrils from which the filaments of the primary array have been removed by myosin extraction (Fig. 33), this elongated central zone of the sarcomere appears to be empty, but it is probable that we have not succeeded in preserving the structures that presumably exist there in the living fibril. Fibrils from which myosin has been extracted are very easily stretched, and shorten again to somewhat less than rest length when released. The components that stretch must be located in this central zone of the sarcomere, for during stretch there is no measurable change in the length of the denser part of the fibril backbone, which reaches from the edges of the gap to the *Z* lines. It is plausible to suggest that very fine extensible filaments, the *S* filaments, bridge the gap and connect the two sets of relatively inextensible actin filaments attached to the *Z* lines. Our belief that the actin filaments do not stretch is also supported by preliminary observations on the fine axial periodicity sometimes seen in electron micrographs (Fig. 6); the spacing of these fine striations in both the *I* band and the *A* band is not changed by stretch nor, apparently, by shortening down to about 65% rest length. Similarly, low-angle X-ray diffraction photographs of frog sartorius muscle fibres stretched to 140% in Ringer solution show the same axial periodicity (415 Å.) as in rest-length fibres (Huxley, 1951, 1953*b*); furthermore, although only very poor X-ray diagrams were obtained from contracted muscle, no changes in the axial period were observed.

The *S* filaments are apparently held under tension in a rest-length muscle, for, when muscle fibres are allowed to shorten spontaneously to equilibrium length (about 80% rest length) before glycerol extraction, their fibrils are found to lack *H* zones, and their *I* bands are correspondingly shorter.

We conclude that during extension of plasticized fibrils actin filaments are drawn out of the array of myosin filaments in the *A* band, thereby stretching the *S* filaments. The actin filaments themselves do not extend, and there is no change in the length of the myosin filaments.

VI. DISCUSSION

(*a*) *General*

As a basis for discussion, the results we have described are summarized in diagrams (Text-figs. 3–8) which show the arrangement within the sarcomere of the three different types of filaments—actin filaments, myosin filaments and *S* filaments. The myosin filaments do not change in length during stretch, during isometric contraction or during isotonic shortening within the normal physiological range, namely down to 65% rest length (although, of course, there may well be internal structural changes in the filaments which leave their overall length unaltered). The significant structural changes taking place during extension or contraction of a muscle are, we consider, as follows. When an unstimulated muscle is stretched, the actin filaments are pulled part of the way out of the *A* band, i.e. out of the interstices of the array of myosin filaments; the actin filaments do not change in length, but the *S* filaments in the centre of the sarcomere are stretched. During isotonic contraction, a translation of actin filaments *into* the array of myosin filaments takes place, and with increasing shortening of the sarcomere the process is accompanied (as indeed it must be for steric reasons) by shortening of the actin filaments themselves, particularly at certain places along their length. During isometric contraction (i.e. when the length of the sarcomere as a whole remains the same, the load being sufficient to prevent shortening), the actin filaments move a short distance into the *A* band, and the *S* filaments shorten; at the same time a series elastic component (possibly located where the actin filaments join the material of the *Z* line) is stretched.

Before going on to more detailed considerations, we should point out that this scheme goes part of the way, at least, towards providing plausible reasons why it may be advantageous for some types of muscle to be cross-striated. The appearance of cross-striations is really the outcome of two separate features; first, that both the actin molecules and the myosin molecules are organized into filaments which do not extend continuously throughout the whole length of the muscle; and secondly, that the actin filaments and the myosin filaments are arranged in register, so that they form groups which are visible in the light microscope. Thus, one might imagine a structure where the discontinuous distribution was present, but not the alignment; perhaps smooth muscle has such a structure!

The first feature, the fact that the total length of either the actin filaments or the myosin filaments is less than the length of the muscle, has the following very obvious advantage: it allows the muscle to shorten for a short distance—over its most important range, in fact—without either the

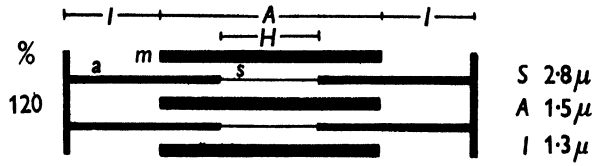


Fig. 3.

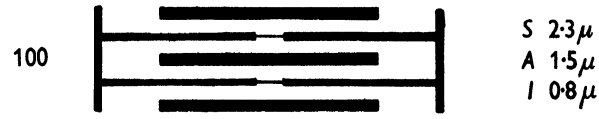


Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

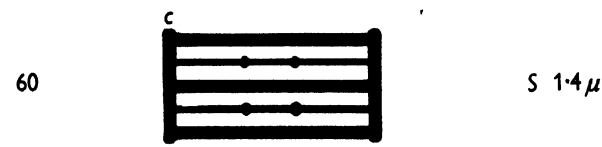


Fig. 8.

Text-figs. 3–8. Diagrams of the arrangement of filaments in one sarcomere of a myofibril. Fig. 3: stretched to 120% rest length while plasticized. Fig. 4: at rest length. Fig. 5: in isometric contraction. Fig. 6: contracted to 90% rest length. Fig. 7: to 80% rest length. Fig. 8: to 60% rest length. Sarcomere lengths (*S*) and *A* and *I* band lengths are given to the right of the diagrams, and lengths of sarcomeres expressed in % rest length are given to the left. *s*, '*S* filament'; *a*, actin filament; *m*, myosin filament; *s.e.c.*, series elastic component; *c*, contraction band. No attempt has been made to illustrate in these diagrams the changes in fibril diameter that accompany the changes in length.

actin or the myosin filaments having to be folded or distorted. Furthermore, the muscle can shorten over the whole of its usual physiological range without distorting the myosin filaments; it is not improbable that any externally imposed structural disturbance of the myosin filaments would adversely affect the operation of the enzymatic sites on the myosin molecules responsible for energy release during contraction.

The second feature, the arrangement of the actin filaments and of the myosin filaments in register, has also obvious mechanical advantages; the only relative movement that takes place between adjacent filaments is that movement which is *necessary* for contraction—the movement of actin filaments past myosin filaments. At no point in the muscle are there myosin filaments sliding past other myosin filaments, or actin filaments past other actin filaments, as would be the case in an unaligned system. Thus internal frictional losses are reduced to a minimum: the whole process is extremely well ordered. Until more is known of the detailed mechanism of contraction, no quantitative explanation can be given for the length of the sarcomere in cross-striated muscle; but it may be pointed out that with a given velocity (v) with which an actin filament can slide past a myosin filament, then the velocity of shortening of unit length of muscle (V) will be determined by the number of sarcomeres (n) per unit length.

$$V = vn = \frac{v}{s},$$

s = sarcomere length.

There may well be, of course, other and more important reasons why the existence of cross-striations is commonly associated with speed and efficiency, for example, factors that are concerned in transmitting stimulation from the surface of the fibre to the contractile fibrils within it; but discussion along these lines lies outside the scope of this paper.

Turning now to more detailed consideration of our results and of previous work, particularly of results obtained by studying actin and myosin *in vitro*, we are now in a position to discuss the probable behaviour of these proteins *in vivo*, and to formulate some new ideas about contraction and stretch in striated muscles. The mechanism of stretch appears to be easier to understand, and we shall discuss it first.

(*b*) *Stretch*

It is well known that an unstimulated muscle fibre is readily extensible, and we have given evidence that in such a fibre actin filaments are apparently able to slide past the myosin filaments and so be pulled part of the way out of the A band. On the other hand, a fibre in the state of contrac-

n (Hill, 1950), or in rigor, is relatively inextensible, and we have found ıt in these cases the actin filaments are unable to move relative to the 'osin filaments. This suggests that there are now cross-linkages between ıse two kinds of filaments, and that these linkages are absent in relaxed ıscle. Results obtained by low-angle X-ray diffraction and electron croscopy on muscles at rest length support this suggestion; they indicate ıt the positions of the actin filaments amongst the myosin filaments are bilized in some way when the muscle is in rigor, whilst in a relaxed ıscle the actin filaments move more freely. The available biochemical ormation agrees with this picture very well. Probably the most significant ference between muscle in the state of rigor or contraction and muscle the relaxed state is in the ATP concentration in the immediate vicinity the filaments. It is widely accepted that the ATP present in a relaxed ril is dephosphorylated when the fibril contracts or passes into rigor. It ılso thought that actomyosin *in vitro* is dissociated into actin and myosin der the influence of ATP, and that the two components recombine when TP has been dephosphorylated. Thus one would expect links to form tween actin and myosin in muscle when the ATP concentration was vered; and if these proteins are organized into separate filaments in the y we have described, then such links will strongly inhibit the extensi- ity of the muscle. This concept provides a structural interpretation of ; plasticizing action of ATP studied by Weber and his colleagues 'eber & Portzehl, 1954).

The elements which are responsible for the long-range elasticity of ıscles in the absence of actin-myosin linkages appear to be the *S* filaments the *H* zones of the fibrils. Both their structure and their chemical ıntity are at present entirely unknown.

(*c*) *Contraction*

The central feature which these studies on striated muscle reveal is that, er a substantial range of lengths (90–140% rest length), contraction and 'etch are brought about by two overlapping sets of filaments sliding past ch other, the length of the filaments themselves remaining approximately nstant. Appreciable shortening of the filaments only occurs during con- ıction to shorter lengths and is therefore not a necessary concomittant of ; basic process of contraction; in fact, it may merely be the *result* of it. ıis concept represents a substantial departure from the ideas held in the st, when it was almost invariably assumed that the cause of contraction ıst be the large-scale shortening of the filaments themselves, brought out by some internal folding mechanism. It is of some interest, there- re, to see whether this new concept, in combination with other recently

obtained structural information, can throw any fresh light on the mechanism of contraction.

It is profitable first of all to consider some of the quantitative relations involved. From the biochemical estimates of the quantities of actin and myosin present in muscle (Hasselbach & Schneider, 1951), knowing their molecular weights (Tsao, 1953; Weber & Portzehl, 1952), and the filament separation and arrangement (X-ray data—Huxley, 1951, 1953*b*) and filament and sarcomere lengths (phase-contrast microscope data), one may calculate the number of molecules of actin and myosin present in each of the two kinds of filaments in each sarcomere.* The calculations show that there are approximately 450 actin molecules in the two actin filaments (each 1μ long) extending from the *Z* lines as far as the *H* zone in the rest-length sarcomere, and approximately 230 myosin molecules in each myosin filament (1·5μ long) in the *A* band. (This calculation does not use the electron-microscope values for filament diameters, which are susceptible to artifact.) If these molecules are arranged in some regular manner along the filaments, then in a rest-length sarcomere there will be, on the average, one actin molecule every 44Å. and one myosin molecule every 65·4Å., although of course the actual repeating period may be a higher multiple of these values if there is more than one molecule in the cross-section of a filament; it cannot be less.

The number of cross-linkages which form between myosin and each 70,000 Mol.wt. unit of actin is likely to be small; the figures of Mommaerts (1950) would indicate that there is only one link. Thus the average axial separation between links must be of the same general order of magnitude as the actin period. Such a structure will return to virtually the same configuration each time the actin filament slides along a distance of one actin period. It seems highly likely therefore that contraction will take place in a series of discrete steps, whose length will be the same as the repeat period in the actin filaments, or some small multiple thereof.

The next piece of information one should consider is that the maximal tension per unit area exerted by a contracting muscle near rest length is about 4 kg./sq.cm. From this figure, and the X-ray information on filament arrangement, it is possible to calculate that the tension in each actin filament is $3{\cdot}3 \times 10^{-5}$ dynes. Thus if the energy for the work done during shortening is derived from the splitting of ATP (10,000 cal./mole), then approximately 1 molecule of ATP would be split for every 2Å. of shortening.

* Rabbit psoas muscle has very little connective tissue or sarcoplasm, and we consider that for the purpose of these calculations we are justified in assuming that the percentages of actin and myosin in whole muscle are virtually equal to their percentages in the myofibrils.

We will now consider what implications these two kinds of numerical results—which have been deduced in a quite straightforward manner from reliable data—may have. The most obvious feature is that the amount of shortening per molecule of ATP split is a great deal less (20–30 times) than the repeat period of either actin or myosin. This is of cardinal importance, for it means that 20–30 molecules of ATP, or some multiple thereof, will have to be split for each step of the shortening process. It becomes apparent, therefore, that if the energy of the ATP molecule is fed into the system in some way by the breaking and reforming of the actin-myosin links, then a considerable proportion of all the links on each filament must be involved in each step of the shortening. We will discuss later the different types of model which might give rise to such behaviour, but first of all we may attempt to pursue the argument about the details of the structure a little further. It should be realized that these attempts are purely speculative; and also that they do not affect the validity of the arguments about the general types of model which will follow.

It will be remembered that each myosin filament is surrounded by six filaments of actin, and that each of these actin filaments has, in turn, three filaments of myosin around it. It is therefore plausible to suggest that the myosin filament contains six molecules of myosin in parallel, one for each of the actin filaments, and that each actin filament, in the same way, contains three actin molecules in parallel. The repeat period of the myosin would then be ~ 392 Å., a value curiously close to the well-known 400 Å. period of muscle. The actin period would be ~ 132 Å.; perhaps the almost exact thirding here is a numerical coincidence, but it is certainly an interesting one. The dimensions of the actin and myosin molecules may conveniently be considered at this point. G-actin is described in terms of an equivalent ellipsoid with axes 140×28 Å. (Weber, 1950). Such a molecule fits into this particular model of the actin filament rather well. On the other hand, the dimensions of myosin are given as 2300×23 Å. (Weber & Portzehl, 1952); these do not fit the model. However, the lengths of both components of the myosin molecule, L- and H-meromyosin, are in the neighbourhood of 400 Å. (Andrew Szent-Györgyi (1953) gives a figure of 435 Å. for H-meromyosin, and he and Dr D. E. Philpott recently informed us that their revised value for the length of the L-meromyosin molecule is 415 Å.), so it is still possible that the 400 Å. period has some significance in myosin.

One might further imagine that sets of cross-linkages between actin and myosin filaments occurred every 400 Å. (i.e. with the same period as the myosin) and that these gave rise to the 'bridging' appearance of the 400 Å. period in some electron micrographs (Hodge *et al.* 1954*a*). In this case,

only one-third of the actin molecules would be linked to the myosin at any one time; in between the points (every 400 Å.) where the three parallel actin molecules are linked to adjacent myosin filaments there would be two sets, each of three actin molecules, which were not so linked, but which could link up if the filament slid along a distance of 132 or 264 Å. Thus this particular model would contract in steps of 132 Å., and each step would involve three actin-myosin links for each 400 Å. length of actin filament, i.e. about 56 links altogether in one half of the *A* band. This is quite a reasonable value, for on the basis of work done per filament one would expect about 66 molecules of ATP to be split per 132 Å. step. Alternatively, one can construct other models in which the step distance is longer (e.g. ~400 Å.) and where a higher proportion (e.g. 100%) of actin molecules are linked to myosin at any one time. Perhaps we should emphasize again that we have carried this calculation through purely by way of illustration, to show that a plausible model *can* be constructed in this manner. This is not to say that the model is the right one, but merely that this type of model is not obviously wrong.

The mechanisms of contraction which suggest themselves may be divided into two categories—those depending on a substantial amount of shortening in a number of different linkages acting in parallel, and those depending on a relatively small amount of shortening in a number of different linkages acting in series. An example of the first category is given by the model in which each myosin-actin linkage can pull the actin filament along a distance of, say, 132 Å. by the contraction of a *branch* of the myosin molecule; the branch would then unhook and extend again until it linked up with the next molecule 132 Å. along the actin filament. These repetitive configurational changes would not, of course, give rise to any overall change in length of the myosin filaments (this type of model was mentioned both by ourselves and by A. F. Huxley & R. Niedergerke in previous papers). Although it is quite a reasonable model, one is a little disturbed by the extent of the configurational change brought about by one molecule of ATP; also, it is a little disappointing in the sense that one has arrived back at 'contractile' myosin again without any reference to the very interesting properties of actin.

The principle of the operation of models of the second category (series addition of small shortenings) is that one of the filaments (e.g. the myosin filament), acts as a fixed support, while the other filament, actin say, still fixed to the myosin filament at one end, undergoes sufficient shortening for its distal end to move along one step: the actin filament then re-links to the myosin in a zipper-like manner, extending slightly in the process, so that at the end of the operation the whole filament has moved along one step.

The actin filament then unzips from its distal end again, shortening slightly in the process, and the whole cycle repeats. Essentially, it is postulated that the actin filament is slightly extended ($\sim 2\%$) when it is bound to myosin, and that the actual pull is exerted when it returns to the normal configuration. It is not difficult to invent mechanisms whereby the *G–F* transformation of actin could be involved in such a process; the most immediately attractive feature, however, is that the amount of movement connected with the splitting of each molecule of ATP is conveniently small.

Perhaps these hypotheses have become rather extended; however, the observational data that led to them are well founded, and if they suggest further lines of experiment, they will have served their purpose.

VII. CONCLUSIONS AND SPECULATIONS

(*a*) *Conclusions*

1. Striated muscle may be considered as a structure built up from two separate sets of longitudinal filaments, one consisting of myosin, the other of actin.

2. The myosin filaments are confined to the *A* bands, where they give rise to the high density and birefringence of those bands. They are spaced out in a hexagonal array 455 Å. apart and their diameter is ~ 110 Å. (probably somewhat more).

3. The actin filaments extend from the *Z* line, through the *I* band and into the *A* band, where they terminate at the edge of the *H* zone. Their diameter is ~ 40 Å. The actin filaments lie in between the myosin filaments in the *A* band, and in the absence of ATP or other 'plasticizer', they occupy fixed positions midway between sets of three myosin filaments, as though they were held in position by cross-linkages.

4. The *H* zone is bridged by very fine extensible filaments of unknown composition (the *S* filaments) which link up the ends of the actin filaments on either side. The *S* filaments are probably permanently cross-linked to the myosin filaments at some point in the *H* zone.

5. When resting or plasticized muscle is stretched, the actin filaments are partly withdrawn from the *A* band, stretching the *S* filaments in the process and leaving a wider *H* zone. The length of the *A* band remains virtually constant, the increase in length appearing only in the *I* band.

6. When muscle in rigor or during strong contraction is stretched, the actin filaments are not withdrawn from the *A* band and the *S* filaments are not extended. However, it is still the *I* bands alone which increase in length, stretch apparently occurring either in the region of the *Z* line or in the actin filaments themselves.

7. When muscle contracts, the actin filaments are drawn into the *A* band. At first the overall length of the actin filament (*Z–H*) remains constant, but when the *H* zone is filled up (~90% rest length) the actin filaments fold up in some way. The length of the *A* bands (i.e. the length of the myosin filaments) remains virtually constant until the *I* bands have disappeared (~65% rest length). The ends of the *A* bands are then in contact with the *Z* lines, and further shortening is accompanied by the formation of contraction bands at these lines, presumably by crumpling of the ends of the myosin filaments. This is probably a non-physiological process, and may be connected with the so-called 'delta-state' (Ramsey & Street, 1940).

8. The essential feature of both plastic stretch and of contraction is the *sliding* of actin filaments past myosin filaments. Such movement is possible only in the absence of *permanent* actin-myosin linkages which would cause the two filaments to stick together (as in rigor). In the case of plastic stretch, the substantial absence of *any* such linkages enables the actin filaments to be drawn past the myosin filaments quite readily. In the case of contraction, the alternate formation and breaking of these linkages causes a directed sliding movement of the actin filaments relative to the myosin filaments.

9. Contraction probably occurs as a stepwise process, the actin filament sliding along a distance equal to the separation of adjacent molecules on the actin polymer (or a small multiple thereof), the general order of magnitude being 50–100 Å. in each step.

10. At each step, a considerable proportion of all the actin-myosin linkages on the filament—if not all of them—are broken and reformed again.

(*b*) *Speculations*

1. Models can be devised in which contraction is caused either by the extensive shortening of a number of linkages acting in parallel (the shortening of each being equal to the step distance) or by minor shortening of a number of linkages acting in series (the shortening of each being equal to the step distance divided by the number of links).

2. These two categories include respectively (*a*) models in which each step of the contraction is produced by a cycle of shortening and lengthening of branches of the myosin filament, acting in parallel, which attach to a point on the actin polymer, pull in and then release again; (*b*) models in which each step of the contraction is produced by a minor cyclic alteration in the separation of adjacent actin molecules, combined with a zipper-like linking and unlinking to myosin.

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ADDENDUM

Since the preceding part of this paper was written, we have made some further observations on the behaviour of muscle and muscle proteins which are particularly relevant to the problems we have been discussing, and we will therefore mention them here.

Firstly, studies have been carried out by one of us (J.H.) on the details of the process of extraction of the *A*-band material. Their purpose was to define in a more systematic way the influence of the ionic strength and pH of the extracting medium, and the effect of plasticizers (agents such as ATP or inorganic pyrophosphate, which seem to act as specific dissociators of the actomyosin complex), and to compare the results with those expected on the basis of the solubility behaviour of myosin *in vitro*. It was found that the dense material of the *A* band *excluding* the central region (i.e. excluding the *H* zone) behaves as one would expect myosin to behave. Thus buffered hypertonic KCl solutions of moderately high ionic strength ($>0{\cdot}5$), in the presence of a plasticizer (to free the myosin from the actin) and magnesium ions, extract nearly all the material of the *A* bands, but leave a residual central line. The extraction is apparently unaffected by moderate variations in pH, similar results being obtained at pH 6·0, 6·5, 7·0, 7·5 and 8·5. Typical and well-known examples of solutions which satisfy these criteria are Hasselbach and Schneider's pyrophosphate-containing solution ($\mu=0{\cdot}68$, pH 6·4) and Guba-Straub solution plus ATP ($\mu=0{\cdot}6$, pH 6·5). The residual dark line in the centre of the sarcomere is extracted by solutions of higher ionic strength, for example by Weber-Edsall solution (pH 7·5) to which 0·01 pyrophosphate (and magnesium ions) have been added as a plasticizer, bringing its total ionic strength to about 0·8; similarly, the 0·1 M pyrophosphate solution we have often employed

has a sufficiently high ionic strength to extract the central region as well as the rest of the dense material of the *A* band. We have presumed that in this central region, the primary filaments are cross-linked to *S* filaments and not to filaments of actin as in the rest of the *A* band, and we must therefore also presume that these cross-linkages are broken by the solutions of the higher ionic strength. It is interesting to note that such solutions in the absence of a plasticizer extract the central region of the *A* band in preference to the rest of the band. A similar extraction of the centre of the *A* band is also accomplished by distilled water instead of strongly hypertonic salt solutions; if distilled water plus a plasticizer is used (low ionic strength), then the whole of the dense material of the *A* bands is extracted.

Attempts have also been made (H.E.H.) to reconstitute muscle in the following way. It was argued that if the 'backbone', left in fibrils after myosin extraction, consists of actin, then it should be capable of picking up myosin from solution in 0·4 M-KCl, for the actomyosin complex is not dissociated at this ionic strength. Accordingly, extracted fibrils were irrigated with such a solution; it was observed that they became progressively denser and denser as the irrigation was continued (over a period of several minutes); the increase in density took place everywhere except in the 'gap' corresponding to the original *H* zone. After irrigation, the myosin solution was replaced by standard salt solution. The addition of 10^{-4} M-ATP then caused these 'reconstituted ghosts' to carry out a remarkable contraction: each segment—extending from the 'gap' (or original *H* zone) to the *Z* line, and on to the next gap—shortened towards the *Z* line; in fibrils which were free to shorten as a whole, the *Z* lines were drawn together in this process; in fibrils which were stuck to the coverslip, large gaps were left between the dark bands now formed around the *Z* lines. Prior to the myosin treatment, of course, the ghost fibrils were non-contractile, and in fact the recovery of contractility could readily be observed on any given fibril. When myosin was added to intact fibrils, their *I* bands became progressively denser, and subsequent addition of ATP produced an abnormal contraction. In other experiments, it was found that rabbit myosin was taken up by myosin-free 'ghosts' of insect myofibrils (from the indirect flight muscles of the blow-fly *Calliphora erythrocephala*), and on treatment with ATP these hybrid reconstituted fibrils contracted in the same way as reconstituted rabbit fibrils.

These observations clearly give strong support to our view that those filaments which extend from the *Z* line to the *H* zone contain actin.

The reaction between 'ghost' fibrils and the components of myosin, light and heavy meromyosin (Andrew G. Szent-Györgyi, 1953), was also

studied (H.E.H.). Szent-Györgyi showed that H-meromyosin would combine with actin, and that it also possessed all the ATPase activity of the original myosin; it differed from myosin, however, in its solubility properties, being soluble over the range of ionic strengths where myosin precipitates. L-meromyosin, on the other hand, whilst possessing no enzymatic or actin-combining properties, has similar solubility properties to myosin. It was found that 'ghost' fibrils, irrigated with a solution of H-meromyosin in 0·1 M-KCl, became very much denser, as they did in myosin solution. On adding ATP, however, the fibrils returned to their original density, without any contraction taking place; presumably, ATP dissociated H-meromyosin from the actin backbone, and the H-meromyosin simply passed into solution again. The presence of L-meromyosin had no observable effect on the behaviour or appearance of the fibrils either without, or with previous, simultaneous, or subsequent, treatment with H-meromyosin.

EXPLANATION OF PLATES

With the exceptions of the fresh *Calliphora* fibrils (Fig. 29) and the minced rabbit muscle (Figs. 12–14) all light micrographs are of glycerol-extracted rabbit psoas fibrils in standard salt solution (described on p. 233) or in the protein-extracting solutions with which they have been treated. All electron micrographs are of thin sections through material that was fixed in buffered osmium tetroxide solution, treated with phosphotungstic acid, and imbedded in methacrylate (for details see Hodge, Huxley & Spiro (1954*a*) and Huxley (1953*a*)).

PLATE 1

Rest-length glycerol-extracted rabbit psoas fibrils

Fig. 1. Fibrils photographed (*a*) in a phase-contrast system, (*b*) in polarized light.

Fig. 2. Longitudinal section of one sarcomere. Electron micrograph.

Fig. 3. Transverse section of one fibril. Electron micrograph. The section has passed through the *A* band to one side of the *H* zone; (*b*) is an enlarged print showing more clearly the double hexagonal array of thick and thin filaments.

PLATE 2

Rest-length glycerol-extracted rabbit psoas fibrils. Electron micrographs.

Fig. 4. Longitudinal section. In the *A* band indicated by an arrow the section shows an array of the thinner filaments. In the other *A* band it includes the thicker filaments as well.

Fig. 5. Transverse section through the *H* zone. At one side of the fibril part of the adjacent *A* band has been included in the section.

Fig. 6. Longitudinal section through a number of fibrils showing, along the whole length of each sarcomere, a fine axial period of approximately 400 Å.

PLATE 3

Fresh frog sartorius muscle.

Fig. 7. Electron micrograph of a transverse section through frog sartorius muscle fixed without prior glycerol extraction. Note *A* bands (double hexagonal array of filaments), *H* zones (single hexagonal array of thick filaments) and mitochondria.

PLATE 4

Fig. 8. Low-angle X-ray diffraction diagram of glycerol-extracted rabbit psoas muscle; slit camera, fibre axis parallel to slit. Two reflexions visible, at Bragg spacings of 395 and 227 Å., i.e. corresponding to $10\bar{1}0$ and $11\bar{2}0$ reflexions from hexagonal lattice with $a = 455$ Å. (± 15 Å.).

Fig. 9. Fourier diagram obtained using reflexions in Fig. 8; hexagonal symmetry was assumed (making phase angles real), with $10\bar{1}0$ positive and $11\bar{2}0$ positive. This diagram represents a possible electron-density distribution in the fibre, seen end-on. The presence of a double hexagonal array of filaments is strongly suggested, a second set of filaments (seen end-on) possibly occurring at specific sites in between the basic hexagonal array of primary filaments 455 Å. apart.

Fig. 10. As in Fig. 8, but using live frog sartorius muscle. The two reflexions visible occur at Bragg spacings of 380 and 220 Å., i.e. corresponding to the $10\bar{1}0$ and $11\bar{2}0$ reflexions of hexagonal lattice with $a = 440$ Å. (± 15 Å.). Notice that while the lattice dimensions are not significantly different from those in Fig. 8, the $11\bar{2}0$ reflexion is very much weaker.

Fig. 11. As in Fig. 9, but using reflexions from Fig. 10 (live muscle); signs as in Fig. 9. While the primary hexagonal array of filaments is still visible, the region in between the filaments is of rather uniform density (cf. Fig. 9), suggesting that the (filamentous) material there is now randomly arranged.

PLATE 5

Myosin extraction by the procedure of Hasselbach & Schneider (1951)
Phase contrast.

Figs. 12–14. Sections through minced rabbit thigh muscles, fixed in 4% formaldehyde, imbedded in paraffin wax and mounted in liquid paraffin after removal of wax.

Fig. 12. Fixed immediately after mincing.

Fig. 13. Fixed after 20 min. extraction, with stirring, in Hasselbach & Schneider's solution (see p. 237).

Fig. 14. Fixed after 5 hr. extraction.

Figs. 15, 16. Rest-length glycerol-extracted rabbit psoas fibrils photographed (*a*) before and (*b*) immediately after treatment with Hasselbach & Schneider's myosin-extracting solution. The bands remaining after treatment are easily identified in Fig. 15.

PLATE 6

Myosin extraction. Glycerol-extracted rabbit psoas fibrils.

Photographs of extracted fibrils in this Plate and in Pls. 7 and 10 are printed so as to give adequate contrast, and the fibrils are in fact less dense than they usually appear here.

Fig. 17. Rest-length fibrils photographed (*a*) before and (*b*) after extraction of myosin by pyrophosphate treatment (see p. 238). Note loss of characteristic *A* bands, and appearance of apparent 'gaps' in positions of original *H* zones. Same fibrils in each photograph. Phase contrast.

Fig. 18. One stretched fibril photographed (*a*) before treatment, (*b*) after extraction of myosin by pyrophosphate treatment, then (*c*) after treatment with potassium iodide solution (see p. 240) to remove actin. After both myosin and actin have been removed, only the *Z* lines and a 'backbone' of virtually zero optical density remain. Phase contrast.

Fig. 19. Longitudinal section of rest-length fibril after treatment with Hasselbach & Schneider myosin-extracting solution. Electron micrograph. Note that array of thick filaments, although no longer visible in most of the *A* band, is still present in the *H* zone.

Fig. 20. Longitudinal section of rest-length fibrils after treatment with pyrophosphate solution. Electron micrograph. Note complete removal of array of thick filaments from whole of *A* band (cf. Fig. 2 and Fig. 19).

PLATE 7

Contraction. Glycerol-extracted rabbit psoas fibrils. Phase contrast.

Fig. 21. The same four sarcomeres of one fibril photographed during step-wise ATP-induced contraction under virtually zero load. Note that while the *A* bands show no changes in length the *I* bands shorten; in (*d*) they have disappeared and contraction bands have formed around the *Z* lines.

Fig. 22. A similar series showing contraction of one short fibril.

Fig. 23. A fibril with contraction bands.

Fig. 24. A rest-length fibril after complete removal of myosin by treatment with pryophosphate solution (p. 238). Note apparent gaps in *H* zone regions.

Fig. 25. A similarly extracted ~90% rest-length fibril. Note absence of 'gaps'.

Fig. 26. A ~75% rest-length fibril (*a*) before and (*b*) after treatment with pyrophosphate solution. Note line in middle of *A* band that remains after removal of myosin.

PLATE 8

Contraction

Fig. 27. Glycerol-extracted rabbit psoas fibrils during step-wise ATP-induced contraction under virtually zero load. Note the changes in the *A* bands while the *I* bands are shortening; the *H* zone disappears and is replaced by a dense line. Phase contrast.

Fig. 28. Glycerol-extracted rabbit psoas fibrils with contraction bands photographed (*a*) in a phase-contrast system and (*b*) in polarized light. The contraction bands are isotropic and the rest of the sarcomere anisotropic.

Fig. 29. A fibril teased out of the indirect flight muscles of a decapitated blow-fly, *Calliphora erythrocephala*, and photographed (*a*) in a phase contrast system and (*b*) in polarized light. Note that this fibril has contraction bands which are isotropic.

PLATE 9

Contraction

Fig. 30. Glycerol-extracted rabbit psoas muscle contracted in ATP. Electron micrographs of longitudinal sections.

(*a*) Very weakly contracted fibrils (long *I* bands), but including some sarcomeres where much stronger contraction has occurred (*I* bands have disappeared).

(*b*) Partly contracted fibrils. Note absence of *H* zones, and compare relative lengths of *I* and *A* bands with e.g. those of the very weakly contracted fibrils in (*a*).

(*c*) Group of fibrils, those on the right strongly contracted, and showing contraction bands, those on the left weakly contracted. Note shortening of *I* bands during contraction, their eventual disappearance, and the appearance of contraction bands where adjacent *A* bands have come into contact with *Z* lines.

PLATE 10

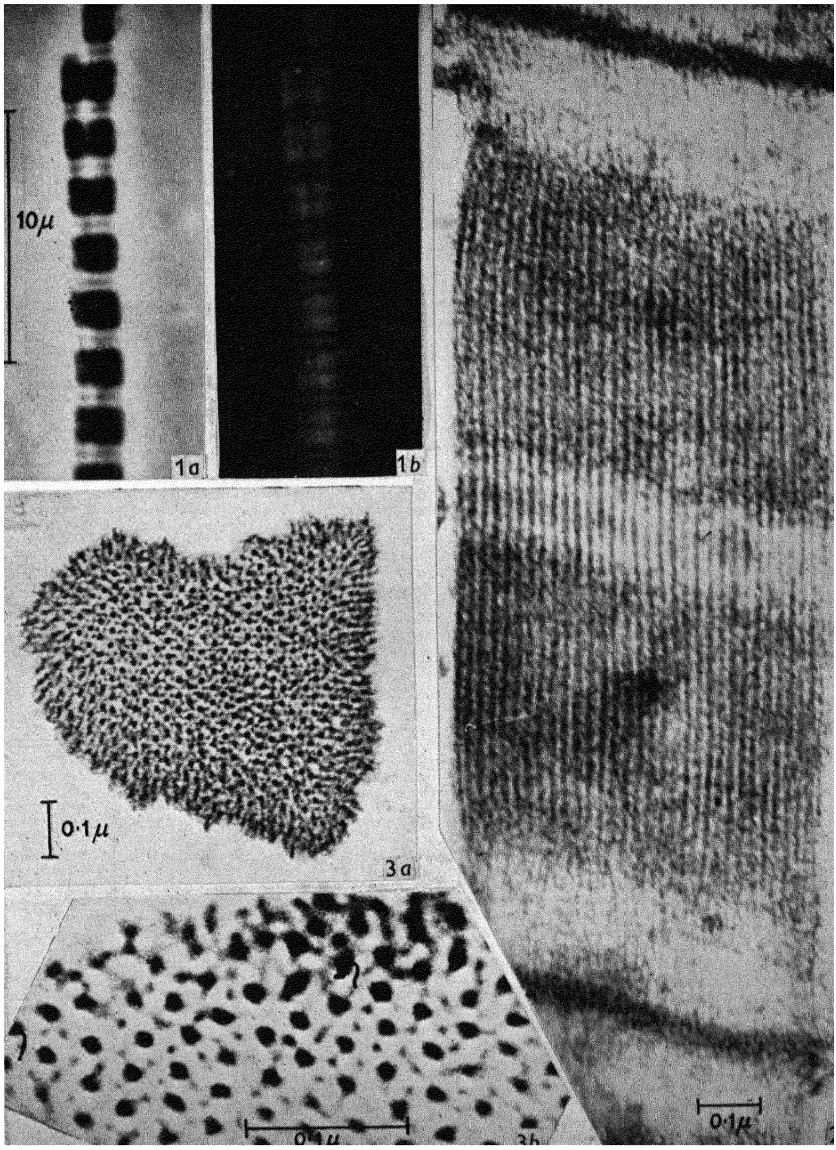
Stretch. Glycerol-extracted stretched rabbit psoas fibrils.

Fig. 31. Electron micrograph of longitudinal section showing long *I* bands and long *H* zones.

Fig. 32. A fibril photographed (*a*) before and (*b*) after extraction of myosin by pyrophosphate treatment. Note correspondence of *Z* lines, and observe long 'gaps' in place of long *H* zones. Phase contrast.

Fig. 33. Fibrils after extraction of myosin by pyrophosphate treatment. Electron micrograph of longitudinal section. Note groups of filaments associated with *Z* lines, separated by wide 'gaps' in place of *H* zones. The material crossing these 'gaps' has presumably not been adequately preserved by osmium tetroxide fixation.

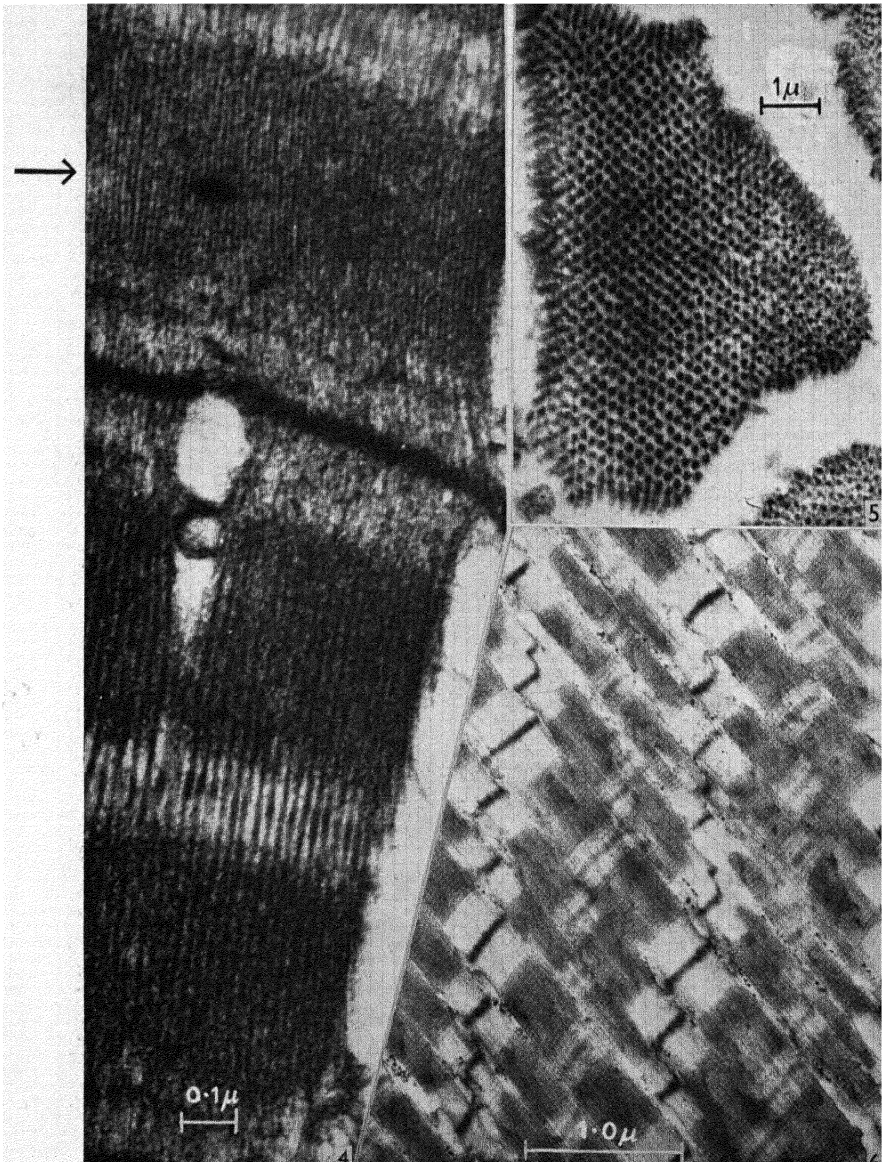
PLATE I



For explanation see p. 262

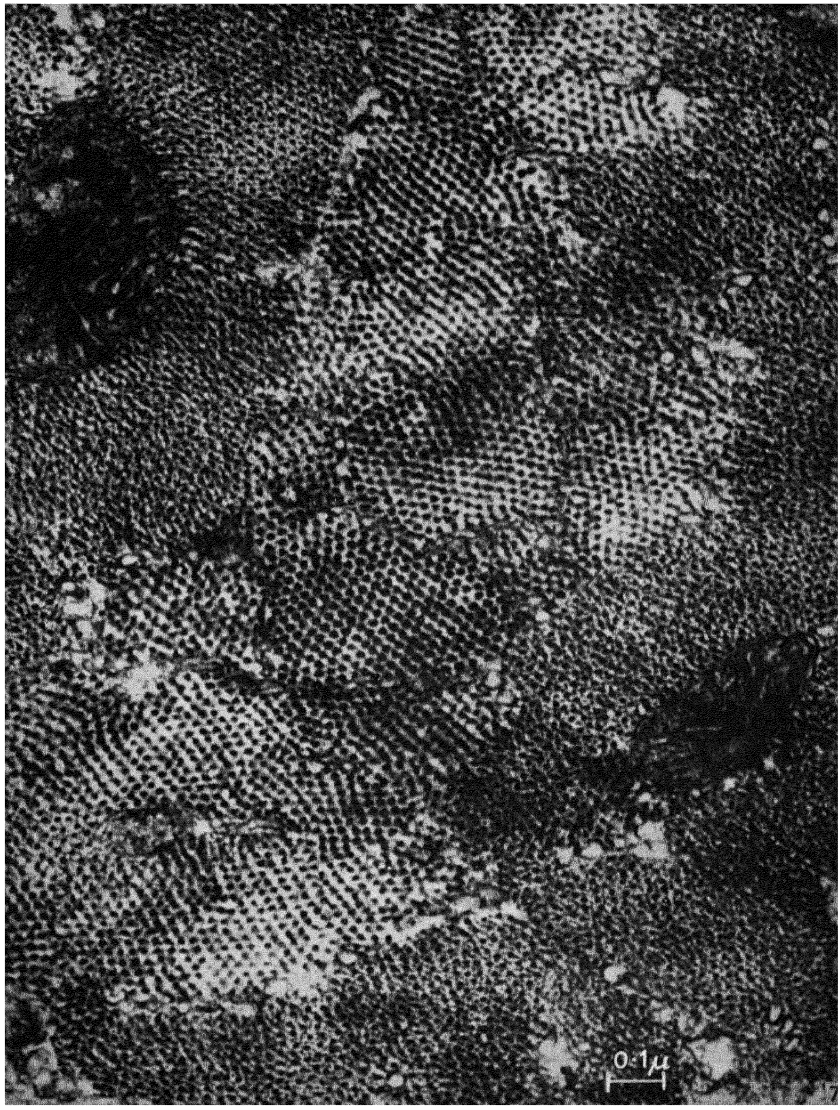
(*Facing p. 264*)

PLATE 2



For explanation see p. 262

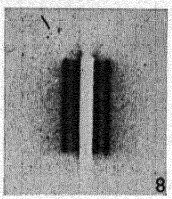
PLATE 3



For explanation see p. 262

(*Facing p.* 264)

PLATE 4



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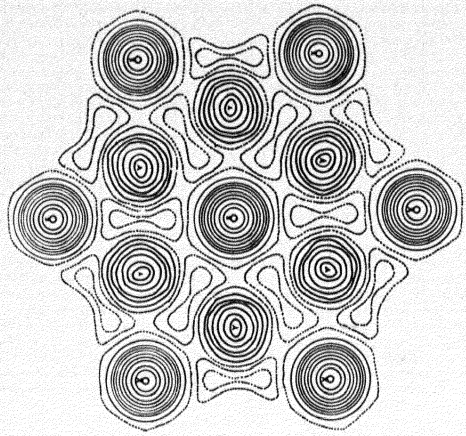
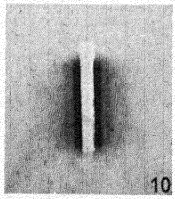


455 Å

9



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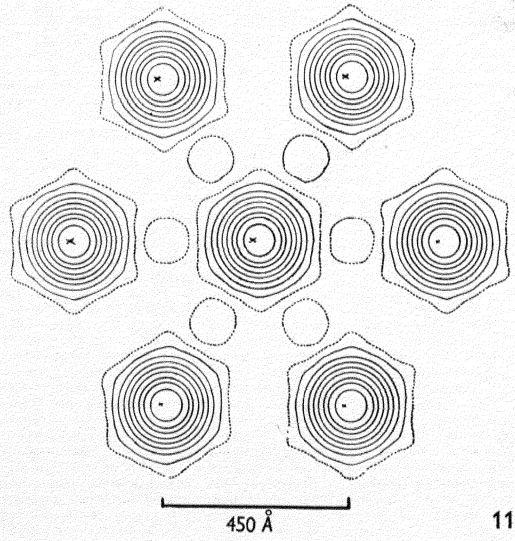
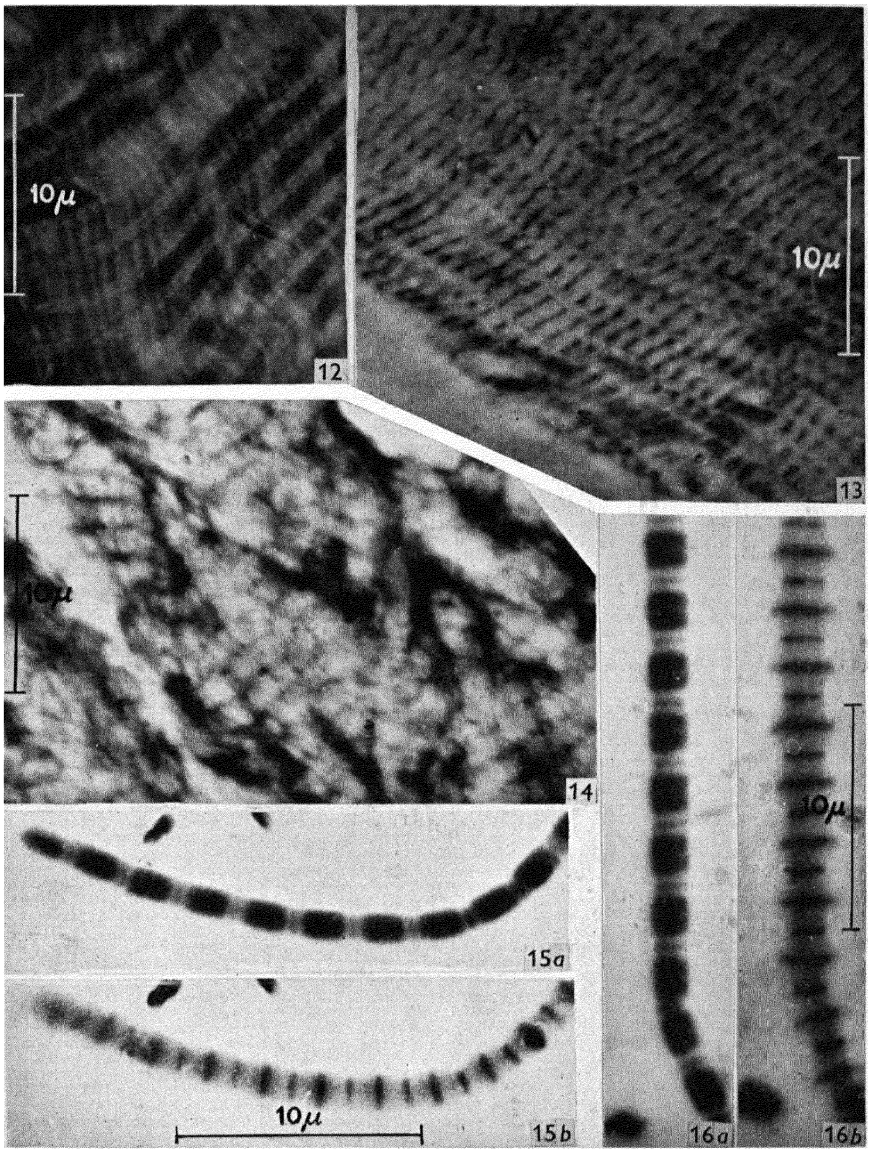


450 Å

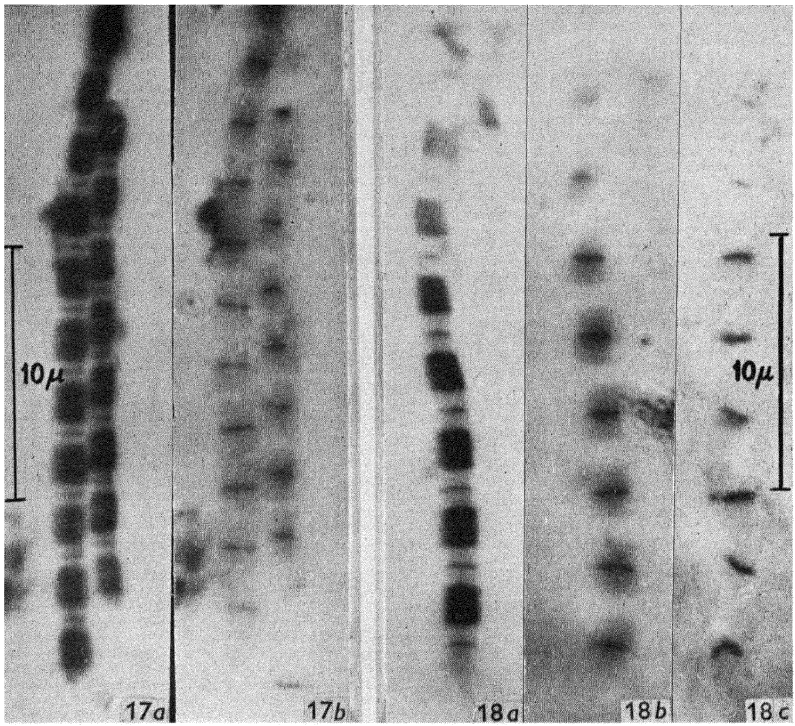
11

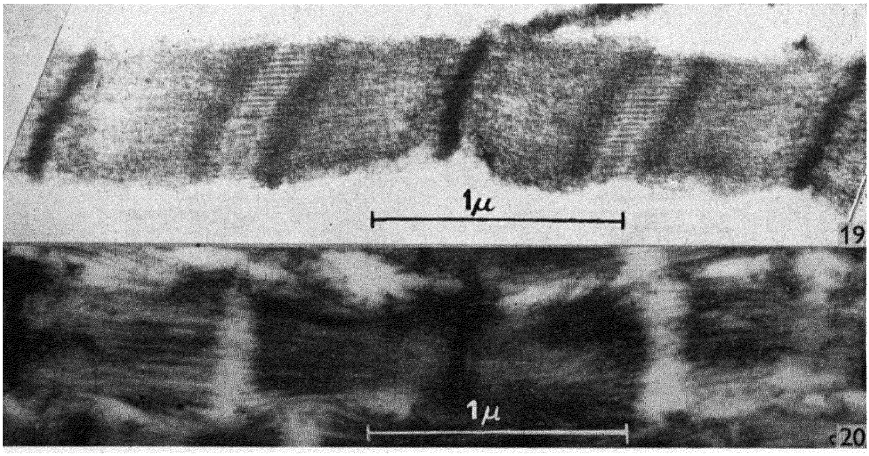
For explanation see p. 263



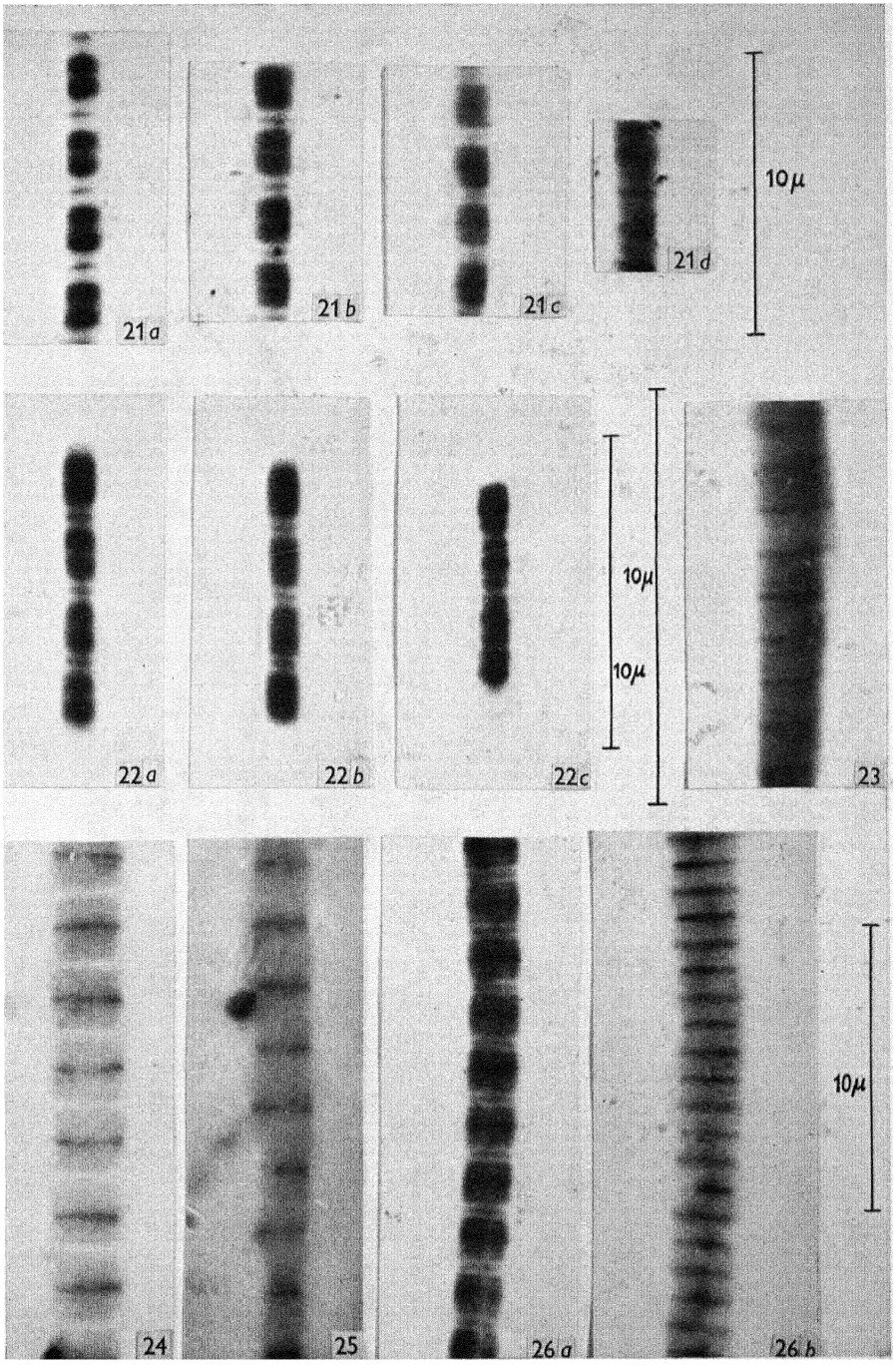
For explanation see p. 263

PLATE 6

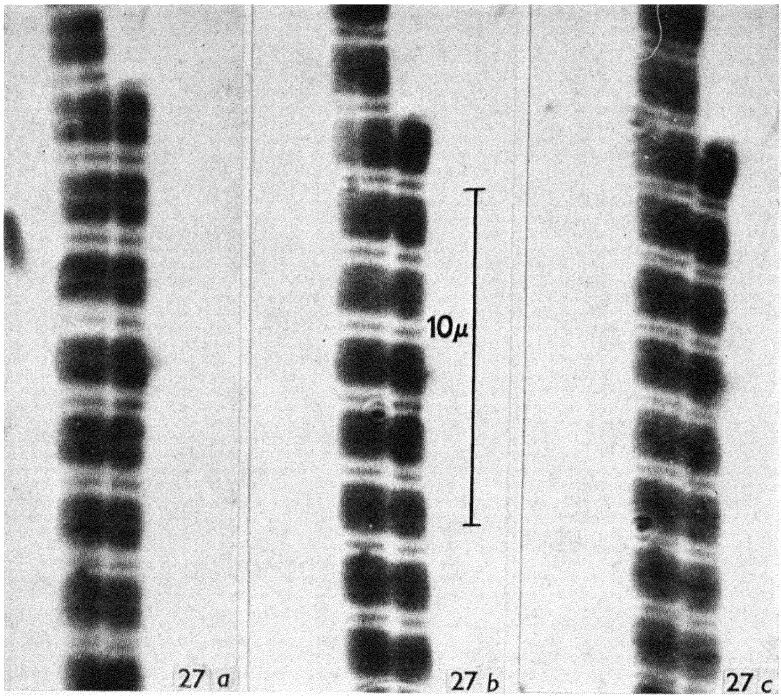




For explanation see p. 263



For explanation see p. 264



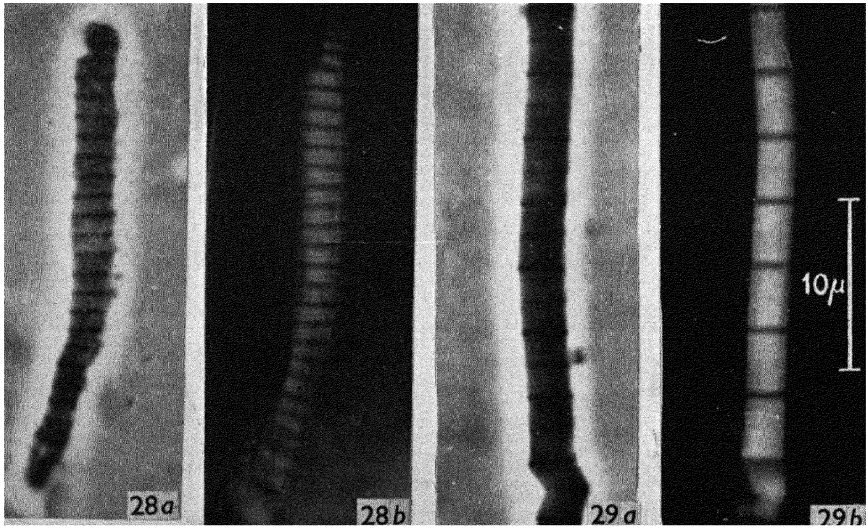


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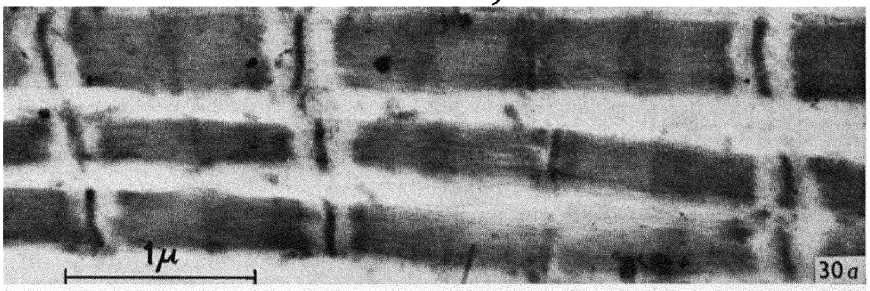
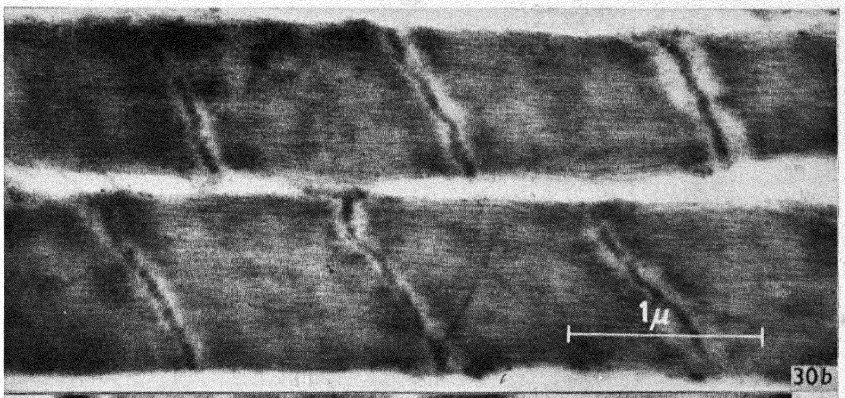
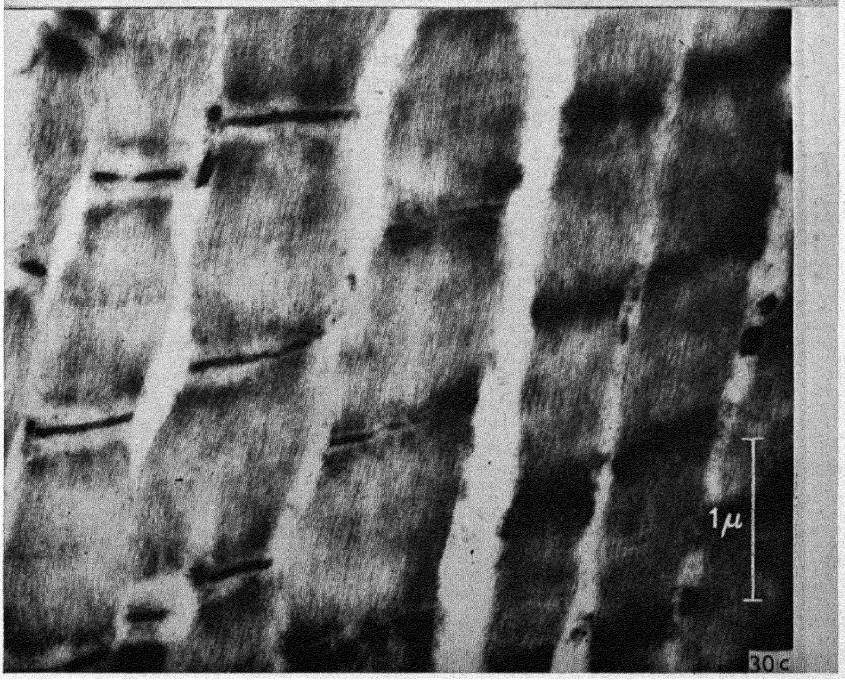
PLATE 9







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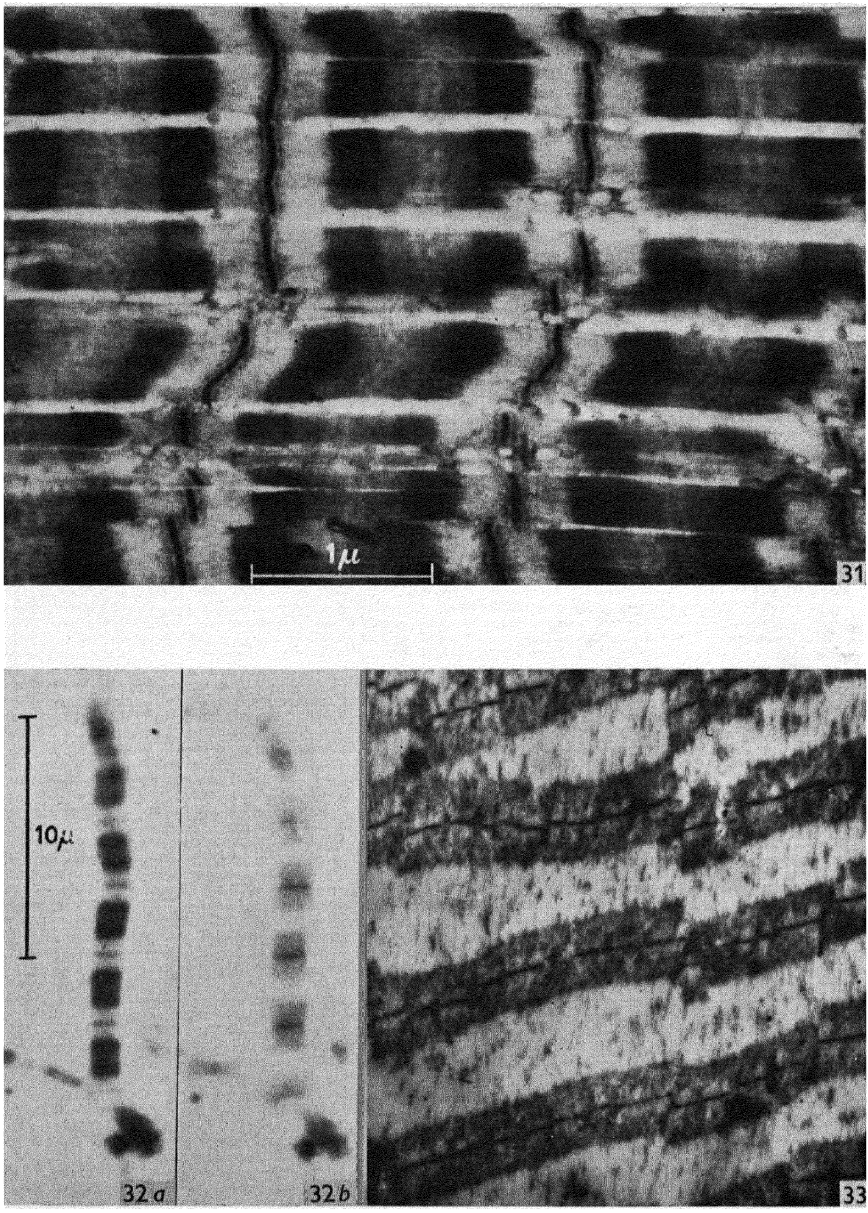

For explanation see p. 264

SOME OBSERVATIONS ON THE INFRA-RED SPECTRUM OF MUSCLE

By B. R. MALCOLM
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INTRODUCTION

In the near infra-red region between 4500 and 4900 cm.$^{-1}$ there are two bands caused by absorption of radiation by the peptide groups in proteins and synthetic polypeptides. By examining these bands with polarized radiation and correlating the results with X-ray diffraction photographs and absorption bands in other regions of the infra-red, it is possible to relate the frequencies and dichroism to the molecular configuration and orientation. Results obtained in this way have been applied by Ambrose & Elliott (1950, 1951*b*) to globular proteins and by Elliott (1952*a*) to an examination of the stretching of hair. These observations have recently been further confirmed by the preparation and X-ray examination of well-oriented and highly crystalline forms of both α- and β-poly-L-alanine (Bamford, Brown, Elliott, Hanby & Trotter, 1954), and their near infra-red spectrum has been obtained by Elliott, Hanby & Malcolm (1954). It is shown here that if the spectra of these model compounds are compared with the spectrum of frog sartorius muscle, limited but quite definite conclusions can be drawn concerning the molecular configuration and orientation in muscle.

EXPERIMENTAL PROCEDURE

On account of the weak absorption of the peptide group in the range 4500–4900 cm.$^{-1}$ compared with its strong absorption in the 3300 cm.$^{-1}$ and 1500–1700 cm.$^{-1}$ regions, it is possible to pass the radiation through a whole frog sartorius muscle. The strong absorption of water makes measurements difficult, but it is sufficiently transparent in a thickness of about 1 mm. for the absorption spectrum of live muscle in Ringer's fluid to be obtained, provided that the muscle is slightly stretched to reduce the scatter of radiation.

The instrument used was a Perkin-Elmer Spectrometer Model A with a lithium fluoride prism. The main details of the techniques used have been described elsewhere (Elliott *et al.* 1954); however, on account of the strong absorption of water some refinements were necessary. Scattered non-monochromatic radiation from the spectrometer is seldom troublesome in

this region, but the transparency of water at shorter wave-lengths, compared with the range under examination, necessitated the use of filters to remove stray radiation of shorter wave-lengths. For this purpose pure germanium is very suitable since it absorbs strongly above 5000 cm.$^{-1}$

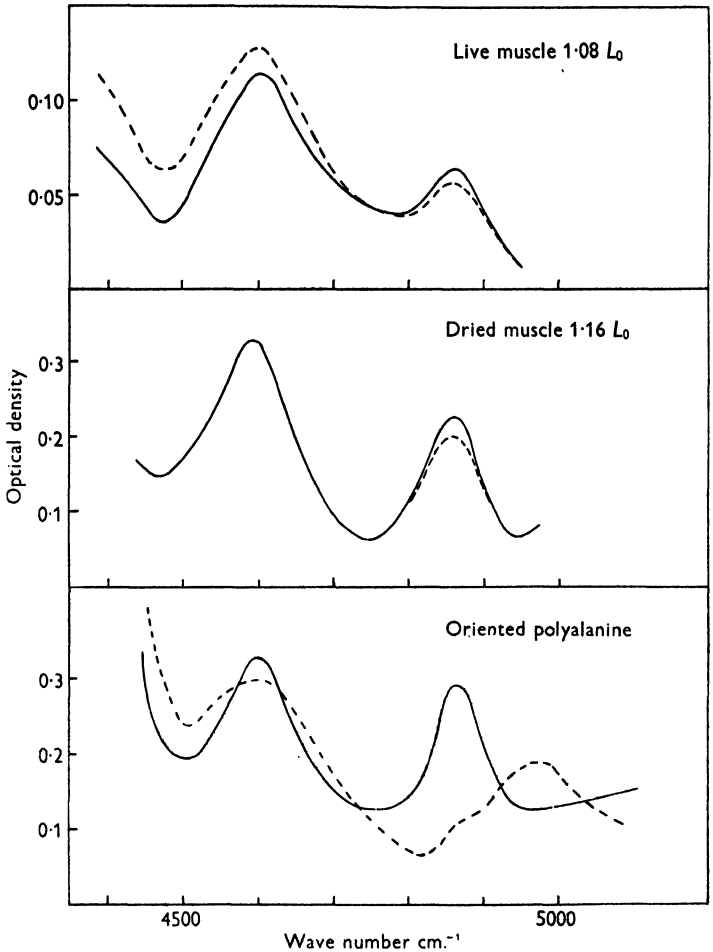

Fig. 1. Above: spectra of live resting muscle and dried muscle stretched beyond the rest length L_0 in the living animal. Below: spectrum of an oriented α polyalanine fibre. Broken line indicates measurements with the electric vector parallel to the muscle or fibre axis; full lines, measurements perpendicular to it.

(Becker & Fan, 1949). Three films of germanium prepared by evaporation on glass substrates were used, their thickness being chosen so that the variation of transmission with wave-length on account of interference gave a maximum at 4900 cm.$^{-1}$, thereby avoiding high reflexion losses. To keep the energy fairly constant throughout the spectrum and to compensate for

the edge of the strong absorption band of water at 5200 cm.$^{-1}$, the spectrometer slit was closed from 0·15 to 0·075 mm. in the range 4900–4700 cm.$^{-1}$. With these precautions it was necessary to use a selected lead sulphide cell cooled to $-78°$ C. as the detector to obtain sufficient sensitivity. Finally, it was necessary empirically to correct for the displacement of water by the protein when measuring the absorption relative to an equal thickness of Ringer's fluid. Errors in this have only a slight secondary effect on the shape and position of the protein bands and negligible influence on the dichroism. These difficulties were not encountered in making measurements on dried specimens and the results are very similar. It was nevertheless thought preferable to concentrate mainly on obtaining reliable measurements on live specimens, since results derived from dried specimens are of restricted value.

Results are also shown for a specimen dried under tension so as to produce the maximum amount of orientation. Considerable lateral shrinkage occurred on drying so that the optical density of the dried specimen is much higher than that of the live one. An X-ray photograph of the dried specimen was also obtained with a vacuum camera giving the usual type of α diagram for muscle.

These results are compared with those obtained with a polyalanine fibre with a high degree of molecular orientation, mainly in the α form but containing about 10% of extended β material. This is revealed both by X-ray photographs and by the development of a dichroic shoulder at 4520 cm.$^{-1}$ on the band at 4600 cm.$^{-1}$ in the infra-red spectrum. Since the orientation of the β material is very high and the dichroism is of the opposite character to that of folded α polypeptides, the dichroism of the NH combination band at 4860 cm.$^{-1}$ is somewhat lowered. In order therefore to compare the dichroism of muscle with that of a pure α material, the spectrum of 10% of β-polyalanine has been subtracted so that the dichroic shoulder at 4520 cm.$^{-1}$ is removed.

EXPERIMENTAL RESULTS

The spectra shown are typical of a number obtained. It will be seen that the spectra of live resting muscle and dried muscle are very similar, the main frequencies are the same and the dichroism of the NH combination band at 4860 cm.$^{-1}$ is very low but of the same character as in oriented α synthetic polypeptides. In addition to this, in live muscle there is also considerable form dichroism which disappears on drying. This causes the apparent optical density to decrease towards longer wave-lengths more rapidly when the electric vector is perpendicular to the fibres than when

it is parallel. To minimize the effect of this and also of the possible overlapping of bands causing an uneven background, in measuring dichroic ratios it is necessary to take for zero the average value of the optical densities measured immediately outside the band. This limits the quantitative accuracy, but in many cases rough quantitative measurements are valuable in the absence of more precise methods.

The band at 4600 cm.$^{-1}$ is quite symmetrical and shows no sign of a component at 4520 cm.$^{-1}$ as is found in silk gut (Ambrose & Elliott, 1951*a*) and synthetic β polypeptides. Pauling & Corey (1951) have suggested that muscular contraction involves a transition from β to α forms. They suggest that although Astbury & Dickinson (1940) have been unable to observe by wide-angle X-ray photographs any β material, there may be sheets of β material separated by relatively thick layers of water so that they would not be detectable. The infra-red spectra shown here indicate that there can be very little β material either in the crystalline or semi-amorphous regions and therefore there are no grounds for supposing muscular contraction to be predominantly a β to α transition.

The dichroism of the NH combination band enables the overall orientation of the protein molecules to be measured assuming that the primary molecular fold is the same as in α synthetic polypeptides. Alternatively, if there are crystalline well-oriented regions in muscle with the same molecular configuration as synthetic polypeptides and also an amorphous fraction or regions of secondary folding giving zero dichroism in any primary molecular fold, the ratio of the relative amounts of these two forms may be measured. In the spectrum shown of polyalanine the NH band is highly dichroic (the band at 4970 cm.$^{-1}$ arises from the crystalline regions only and is even more dichroic). If we neglect the effect of amorphous and poorly oriented molecules, that is, assume that all the molecules in the polyalanine are perfectly oriented, the angle θ that the transition moment giving rise to the absorption makes with the molecular axis may be calculated from the dichroic ratio R. This is about 7 if allowance is made for the effect of the crystalline band which interferes with the background. In the case of muscle, both live and dried, the dichroic ratio is about 1·2. These results may conveniently be expressed in terms of a fraction F of the molecules being perfectly oriented and $(1-F)$ randomly oriented or in some type of secondary folding in which the dichroism is zero. It can be shown (Fraser, 1953) that

$$R=\frac{\frac{1}{2}F\sin^2\theta+\frac{1}{3}(1-F)}{F\cos^2\theta+\frac{1}{3}(1-F)}.$$

For polyalanine substituting $F=1$ and $R=7$, θ is found to be 75°. Using this value for muscle and substituting $R=1{\cdot}2$, a value of 0·15 is obtained

for F. That is, assuming that the primary fold of the protein molecules in muscle is the same as in polyalanine, a maximum of only 15% can be considered as oriented parallel to the muscle axis, the remainder being folded in secondary folds or amorphous, giving zero dichroism.

This contrasts with the conclusion drawn from wide-angle X-ray studies by Astbury (1947), who suggests that the α chains in proteins such as hair and muscle are well oriented. The limitations in the value of the X-ray method alone have been pointed out by Elliott (1952*a*) in relation to the stretching of hair, and similar considerations apply to muscle. The sharp reflexions on wide-angle X-ray photographs arise from the crystalline regions, which are much more highly oriented, and cannot be taken as a measure of the overall orientation.

Huxley & Perutz (1951) have found a meridian reflexion at 1·5 Å. in dried muscle. A preliminary inspection did not reveal a similar reflexion off the meridian. This is characteristic of α helices as found in α-polyalanine and is consistent with the character of the dichroism in muscle. These results suggest that the oriented component in muscle may contain α helices, but as yet there is no evidence that the remaining 85% of the muscle is in this configuration; in fact, the extreme rigidity of the helical structure makes it very difficult for it to be incorporated in any type of secondary fold.

An alternative conclusion which might be drawn is that the primary molecular fold is different in muscle from the α helix as exemplified by polyalanine and that it is in fact well oriented. Very little dichroism in single fibres of muscle in the region of fundamental vibrations has been found by Morales & Cecchini (1951), which again contrasts with oriented synthetic polymers, and these authors conclude, in agreement with the results here, that the order in muscle exists at a larger scale.

It is interesting to note that from an infra-red standpoint, the spectra of muscle are very similar to those of regenerated protein fibres (Elliott *et al.* 1954) in which there is again no evidence of marked unfolding into the β form and very little dichroism. The dichroism is usually low, too, in globular protein crystals (Ambrose & Elliott, 1951*b*; Elliott, 1952*b*). In conclusion, it should be emphasized that while infra-red methods in the overtone region can be used to rule out the presence of β structures since there is no evidence of a band at 4520 cm.$^{-1}$, the band at 4600 cm.$^{-1}$ is not necessarily specific to α helices. The form of polyglycine (Elliott & Malcolm, 1954) which appears to be neither a β structure nor an α helix has a band at 4600 cm.$^{-1}$. There may be other configurations, and in particular there is no evidence in proteins that hydrogen bonds are not formed between the peptide group and water molecules, which would confer great flexibility on the structure.

The writer would like to thank Prof. A. V. Hill for his advice and instruction in dissection, and Dr A. Elliott for helpful discussions. Thanks are also due to Mr W. E. Hanby for the polyalanine and Mr L. Brown for taking the X-ray photograph.

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THE LINK BETWEEN METABOLISM AND MOTILITY OF CELLS AND MUSCLES

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I

A. V. Hill (1926*a*) once said that there was some evidence to suggest that the movements of all living organisms are based on the same fundamental process. Direct evidence has been obtained to-day that every mobility of living systems is apparently a function of two different basic mechanisms.

The first of these two mechanisms seems to be identical for every contraction, i.e. for the contraction of all smooth and cross-striated muscles right down to the contraction of individual cells during the movements of mitosis. This mechanism is a complicated reaction of the contractile fibrillar proteins with ATP (adenosinetriphosphate) or with ITP (inosinetriphosphate). Eventually this reaction leads to the splitting off of the terminal phosphate group of the phosphorylated nucleotides (Weber & Portzehl, 1952; Szent-Györgyi, 1953).

The second mechanism produces lengthening movements of cells. They occur especially during the anaphase of mitosis, appearing as the stretching of the whole cell and the spindle apparatus. This, too, is a reaction between protein and ATP under physiological conditions. Yet we do not know how far the proteins involved in the stretching movements are fibrillar proteins. It is certain, however, that the ATP need not be split in order to bring about this lengthening movement. In addition, ATP can be replaced here by inorganic polyphosphates such as pyrophosphate.

The compound movements of certain parts of cells, however, such as those of flagella, consist in a sequence of contractions alternating with elongations. The contraction phases of the rhythmical movements of flagella naturally are based on the same mechanism as the other contractions of cells and muscles.

We are in a position to make such far-reaching statements, because we have learnt how to separate such reactions of proteins on which motility is based from the numerous ones which serve other purposes. Thus the reactions can be investigated alone. This separation can be accomplished by means of models. When these models are prepared the contractile structure remains completely or to a great extent the same as during life if we confine ourselves to killing the living substances such as muscle fibres

(Varga, 1950), cells (Hoffmann-Berling, 1954*a*, *b*) or flagella (Hoffmann-Berling, 1954*c*) by extracting them with water and glycerol. Hereby the membranes are destroyed and all the soluble proteins, and accordingly the majority of enzymes, are eliminated and the crystalloids removed. The lack of membranes renders these models insensitive to processes of excitation. The elimination of operative substances and enzymes removes the normal processes of recovery.

We call such models fibre models or fibril models when we deal with extracted muscle fibres or fibrils. If we are dealing with cells we accordingly name them fibroblast models, trypanosome models, flagella models and so on or generally cell models.

But we term these models thread models when the contractile fibrillar proteins have been dissolved, isolated, purified and finally extruded to threads, the protein particles of which are orientated parallel to the axis of the thread. Up to now thread models have been prepared only from smooth (Dörr & Portzehl, 1954) or cross-striated muscles (Portzehl, 1951; Portzehl & Weber, 1950).

On addition of ATP or ITP the functional structures of all these models behave as if they were alive. They split the operative substance and move. When in interphase, models prepared from expanded fibroblasts contract (Hoffmann-Berling, 1954*a*; Hoffmann-Berling & Weber, 1953) (Pl. 1, fig. 1). Spindle bodies and protoplasm of anaphase models lengthen (Hoffmann-Berling, 1954*b*; Hoffmann-Berling & Weber, 1953) (Pl. 1, fig. 2). The equator of telophase cells constricts so that only a narrow bridge remains (Pl. 2, fig. 3) (Hoffmann-Berling, 1954*d*). The tails of models obtained from spermatozoa or trypanosomes begin to vibrate (Hoffmann-Berling, 1954*c*). The same applies to the separated flagella of spermatozoa (Hoffmann-Berling, 1954*c*). Fibril, fibre and thread models contract.*

It is the specific behaviour of these movements of models alone which would account for an identical behaviour of the functional proteins both in models and in living tissue.

(1) No movements of models can be induced except by polyorthophosphates; moreover, every *contraction* can be initiated only by ATP or the nearly identical ITP (Portzehl & Weber, 1954).

(2) ATP and ITP do not produce any change in shape of any other known protein (Portzehl & Weber, 1954).

(3) All the energy available from the metabolism of living organisms is transferred exclusively to ATP and is kept available in the form of ATP only.

* All these movements are shown in a microfilm which can be ordered from the Institut für den wissenschaftlichen Film, Göttingen, Germany (Hoffmann-Berling & Portzehl, 1954: Movements of extracted cells and muscle fibrils (models) produced by ATP).

With regard to electrostatic theories of muscular contraction discussed again and again I would point out that no changes in length occur even when the electrical charge is changed as extensively as it is by variations of pH.

II

Models and living substances show a striking uniformity of behaviour not only qualitatively but also quantitatively.

Table 1 indicates that the maximal active tension developed by fibre models prepared from smooth or cross-striated muscles is similar to the maximal tetanic tension reached by the corresponding muscles. This is the case, even though the maximal tension produced by the weakest muscle investigated and by its fibre model (cow rectum) is about one order of magnitude smaller than the maximal tension developed by the skeletal muscle of Mammalia and the fibre model prepared from it.

Table 1. *Active maximal tension* (kg./cm.^{2})

Kind of muscle	Fibre model	Living
Skeletal muscle:		
Rabbit	$4^{(1)}$	$5^{(2)}$
Frog	$3^{(2)}$	$2^{(2)}$
Smooth muscle:		
Anodonta adductor: yellow	$5^{(4)}$	$4{\cdot}5^{(2)}$
white	$2^{(4)}$	
Cow rectum (longitudinal muscle)	$0{\cdot}6^{(4)}$	$0{\cdot}7^{(2)}$

References: (1) Weber (1951); (2) Pauschinger & Ulbrecht (unpublished); (3) Portzehl (unpublished); (4) Ulbrecht & Ulbrecht (1952).

Also the dependence on temperature of the isometric tension is identical for the individual muscle and the corresponding model, though the dependence on temperature is widely different for different kinds of muscles (Weber, 1953).

Thus, the active tension produced by the skeletal muscle of Mammalia and its models decreases to one-fourth (Weber, 1953) when the temperature falls from 20 to 0° C., while the active tension reached by the frog muscle and its models diminishes to four-fifths only (Weber, 1953). Hence, in transition from muscle to model we find retained even the force peculiar to the species to which the individual muscle belongs.

Unlike the maximal tension, the degree of maximal shortening developed by every contraction of models is identical and very high. It is 80–90% of the initial length, whether we regard the contraction of the cell equator of the model during mitosis or the contraction of the fibre or thread model obtained from the skeletal muscle of vertebrates (Table 2). The same high

degree of maximal shortening can always be seen when working with living cells and smooth muscles, although, in the case of living skeletal muscles, only when in the so-called delta-state into which every living skeletal muscle can easily be passed (Ramsey & Street, 1940; Ramsey, 1947).

Table 2. *Maximal shortening of vital and model contraction as percentage of standard length*

Kind of tissue	Model thread	Model fibre	Living
Fibroblasts:			
Cells during interphase	—	~80[1]	~80
Equator of cell during cleavage	—	~70[2]	>80
Skeletal muscle:			
Rabbit	~70[3]	~85[4]	~40
Frog	—	~85[5]	~40
Smooth muscle:			
Anodonta: adductor	—	>80[6]	>80
retractor pedes	~80[7]	>70[6]	—
Cow rectum (longitudinal muscle)	—	~80[6]	—

References: (1) Hoffmann-Berling (1954*a*), Hoffmann-Berling & Weber (1953); (2) Hoffmann-Berling (1954*d*); (3) Portzehl (1951), Portzehl & Weber (1950); (4) Weber (1951); (5) Portzehl (unpublished); (6) Ulbrecht & Ulbrecht (1952); (7) Dörr & Portzehl (1954).

In order not to bore you with detailed comparisons I want to add only the following. Model contraction exhibits the same characteristics which are peculiar to muscular contraction and which have never been observed elsewhere, such as the quick-release phenomenon (Gasser & Hill, 1924; Hill, 1926) and the velocity-load law (Fenn & Marsh, 1935; Hill, 1939). Therefore it is justifiable to say that the process of contraction is changed to a remarkably small extent by its isolation.

III

The model systems are so simple that the conditions of motility can be analysed without great trouble. The investigator creates the model system by adding substances to the model necessary to make the dead model operate. Consequently, he knows the substances added and their concentrations. We know that in order to work the contractile system requires both the protein existing in the model and the operative substances ATP or ITP. But, in addition, we know that three other conditions must be fulfilled:

(1) The reaction of the system must be neutral.

(2) The ionic strength must approximate to the physiological value.

(3) The model system must contain traces of magnesium ions—and in certain special cases also of calcium (Marsh, 1952; Bendall, 1952; Hassel-

bach & Weber, 1953). The numerical value of the concentration of magnesium required seems to be the same for all model systems ranging from cell models (Hoffmann-Berling, 1954*a*) to fibre models from skeletal muscle (Portzehl, 1954). But the concentration of magnesium has to be about one hundred times as great (namely, M/100 Mg) (Hoffmann-Berling, 1954*a*; Spicer & Bowen, 1951) when ITP is applied as operative substance instead of ATP. The latter can be applied in an optimal way even if the concentration is as low as M/10,000 Mg (Hoffman-Berling, 1954*a*; Spicer & Bowen, 1951).

The ionic requirements just discussed are naturally fulfilled in living tissues where salts and buffers are in a physiological concentration. On the other hand, however, analysis shows that there is no specific action of potassium ions on the process of contraction. In the living tissues potassium is in ample quantities. It is apparently required only for the production of membrane potentials, i.e. for the process of excitation.

If a model system is complete but lacking only in ATP or ITP, the protein structure of the system is as rigid as the protein structure of a muscle in rigor mortis (Portzehl, 1952). For the living muscle becomes likewise rigid when its ATP has completely or almost disappeared. Rigidity means high resistance to stretching.

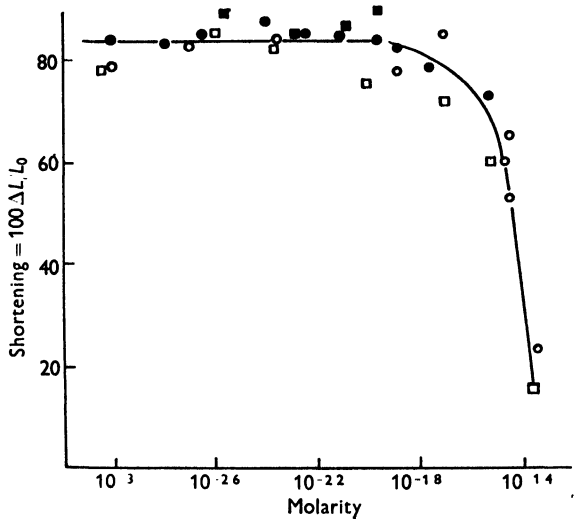
If an optimal concentration of ATP or ITP is present, for example, between 10^{-3} and 10^{-2} M, these operative substances are split by the contractile protein of the models, while the fibrillar protein itself passes into a state of maximal contraction. At the same time the resistance to stretching diminishes to about 40% of the value found in rigor (Weber, 1951; Portzehl, 1952).

If the concentration of ATP and ITP is increased beyond 10^{-2} M, both the rate of splitting of ATP and the magnitude of contraction fall steeply—down to values near zero (Text-fig. 1). It has long been known in enzyme chemistry that the interaction between protein and substrate is rediminished by over-optimal substrate concentrations.

Text-fig. 1 indicates, also with respect to the quantities, the identical action of ATP and ITP on the contractions both of cell and muscle. The numerical value of the concentration of the operative substance influences identically the degree of shortening developed by both cell models and fibril models, independently of whether this operative substance is ATP or ITP. This fact impressively bears out the identity of the fundamental process underlying contraction throughout the realm of life.

The resistance to stretching exerted by the protein structure diminishes further in the range of over-optimal concentrations of ATP or ITP. This resistance to stretching, however, can be brought to very low values also in

the optimal range of the concentrations of ATP or ITP if the splitting is inhibited (Hasselbach & Weber, 1953). This can be achieved either by poisons (Portzehl, 1952) such as salyrgan, which Kuschinsky & Turba (1950*a*, *b*) discovered to be an ATPase poison, or physiologically by the Marsh (1952) factor existing in the living muscle. In this case the model is of just the same plasticity and extensibility as is the living muscle during rest (Hasselbach & Weber, 1953). For during rest also the living muscle



Text-fig. 1. The dependence of maximal shortening of fibril and interphase models on the concentration of the nucleotide. Fibril models: ●, ATP; ■, ITP. Interphase models: ○, ATP; □, ITP. (Hoffmann-Berling, 1954*a*; Portzehl, 1954.)

contains the optimal concentration of ATP and yet does not split ATP—inhibited by its own Marsh factor. The elastic resistance to stretching is the same in rigor, contraction and rest both for the living muscle and the fibre model prepared from it. This can be seen in Table 3.

Table 3. *Resistance to stretch of fibre model and skeletal muscle*

(g.cm.$^{-2}$ L.ΔL^{-1})

State	Fibre model	Skeletal muscle
Rigor	>7500[1]	>6000[2]
Isometric contraction	2200[1]	Between rest and rigor[3]
Rest (relaxed)	20 → 400[4]	20 → 400[5]

References: (1) Portzehl (1952); (2) Bate-Smith & Bendall (1947, 1949); (3) Gasser & Hill (1924), Hill (1926), Buchthal (1942), Reichel (1952); (4) Hasselbach & Weber (1953), Portzehl (1952); (5) Wöhlisch & Clamann (1936), Hill (1949).

Hence, ATP and ITP have a dual action on the contractile protein structure; they render the protein structure plastic and extensible if they are not broken down. They produce contraction if they are broken down. Henceforth the first effect will be referred to as the plasticizing effect.

Contrary to the effect on contraction the plasticizing effect is not strictly specific. For in this reversible plasticizing effect ATP and ITP can be replaced also by other polyphosphates—even inorganic ones.

Plasticizing effect and contracting effect are in reasonable connexion. Should tissues and their models become rigid when the splitting of ATP and contraction cease the contractile structures would not be able to relax.

Apart from this, however, the plasticizing conditions are of independent significance in a special case. Elongation of the cell and the spindle bodies of models in anaphase is brought about either in the presence of ATP even when its splitting is inhibited or in the presence of inorganic polyphosphates (such as pyrophosphate) which are scarcely split by the living tissue (Hoffmann-Berling, 1954*b*). This movement is also specific and cannot be provoked by other plasticizers except by polyphosphates (Hoffmann-Berling, 1954*b*). Hence, there are special protein structures which elongate under the same conditions under which the contractile structures relax only.

IV

Till now we have observed contractions of models only when there is a splitting of ATP (or of the unphysiological ITP). I want to discuss some quantitative results which suggest that it is only the plasticizing effect that is due to a binding of ATP while contraction is exclusively due to a splitting of ATP.

(1) The power of the Anodonta model is about one-seventh as great at 0° C. as at 20° C. Exactly the same is true, however, of the rate of the splitting of ATP (Table 4). It is only in the case of the Anodonta model that the power has been measured at both these temperatures.

Table 4. *Power and rate of ATP-splitting by* 1 *cm.*^{3} *Anodonta model* (G. & M. Ulbrecht, 1953)

	0° C.	20° C.	Proportion
Power (g.cm.sec.$^{-1}$)	9	60	1:7
ATP-splitting (μmol. P.sec.$^{-1}$)	0·028	0·19	1:7

(2) The sole difference between the contraction of the models and that of living systems is to be found in the different speed of shortening provided that the model has been prepared from a tissue which shortens quickly, for

instance, from the skeletal muscle. On the other hand, when living cells or their models shorten, the speed is identical (Table 5). The speed of shortening produced by the living skeletal muscle is nearly 10 times as great as that of its fibre model (Table 5). But it has been found that the rate of ATP splitting by the living muscle is also ten times as great as the rate of splitting by the model (Portzehl & Weber, 1954).

Table 5. *Speed of shortening of contraction (as percentage of standard length per second)*

	0° C.	20° C.	37° C.
Skeletal muscle: Living	~200[1]	—	—
Fibre model	~20[2]	~200[3]	—
Smooth muscle, Fibre model (Anodonta)	~7[4]	~22[4]	—
Cell during interphase: Living	—	—	~0·1[5]
Model	—	—	~0·1[6]

References: (1) Hill (1951); (2) Ulbrecht & Ulbrecht (1954); (3) Portzehl (1954); (4) Ulbrecht & Ulbrecht (1953); (5) Lettré (personal communication); (6) Hoffmann-Berling (1954*a*).

(3) Both the splitting of ATP and the contraction of all contractile structures ranging from the model obtained from the skeletal muscle to the cell model are reversibly poisoned by polysulphonates, for instance, by suramin, and by heavy metal compounds such as salyrgan. The latter compounds block the SH groups reversibly.

In all these instances the plasticizing effect produced by the binding of ATP is completely retained.

(4) Hence it is worth while to inquire about the efficiency yielded by the splitting of ATP and to compare it with the efficiency yielded by the living muscle. The maximal efficiency of the *living* skeletal muscle has been ascertained by A. V. Hill. Hill establishes a relation between power and the heat liberated by the muscle without making any assumption with regard to the source supplying heat and mechanical energy in muscular contraction. If for comparison the maximal power exerted by the fibre model obtained from rabbit is measured and related to the energy liberated by the breakdown of ATP a maximal efficiency of about 40% is found (Ulbrecht & Ulbrecht, 1954). From Hill's measurements maximal efficiencies between 30 and 40% can be calculated for the contraction phase of the living frog muscle. Apparently, then, the splitting of ATP is in fact the source of energy for contraction.

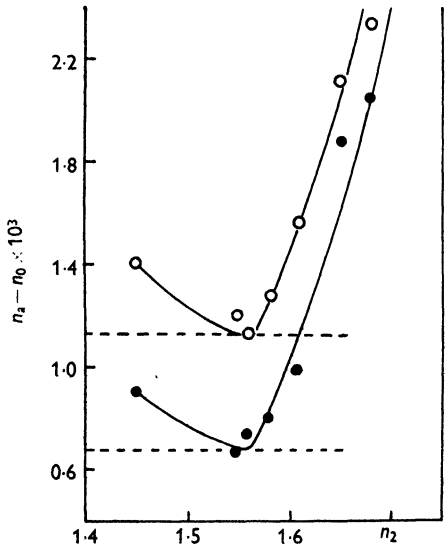
It would be an improbable coincidence if under all these different conditions the binding of ATP, of which both numerical value and speed are

unknown, ran strictly parallel to power as does the splitting of ATP. Therefore the assumption that the binding of ATP renders the protein structure plastic and brings about relaxation while the splitting of ATP produces contraction, appears to be entirely justified.

V

If we acknowledge this we are prompted to ask: How does the contractile structure shorten when ATP is split? Only a few words can be said about this.

Exact data are available for the birefringence of the skeletal muscle and its fibre model. Birefringence of the contractile structure of both tissue



Text-fig. 2. The behaviour of intrinsic and form birefringence during the contraction of the fibre model (Ströbel, 1952). Ordinate: birefringence. Abscissa: the refractive index of the intramicellar fluid. O—O, uncontracted; ●—●, contracted by 70%; ——— total birefringence; – – – – intrinsic birefringence. Contraction at pH ~7; ionic strength ~0·1 μ.

and models is identical. In addition, the birefringence of both decreases also by the same amount during shortening. When working with models, however, this birefringence and its decrease can even be analysed, i.e. the intrinsic and form birefringence can be measured separately (Text-fig. 2). Thus it has been found that the decrease in total birefringence depends on a decrease in intrinsic birefringence while form birefringence remains constant (Ströbel, 1952). The constancy of form birefringence suggests that the orientation of the protein rods of the contractile elements remains parallel to the axis of the fibre during contraction. The decrease in intrinsic

birefringence, however, indicates that the single rods shorten during contraction. Thus the visible contraction is a function of the invisible occurring in the single particles of the contractile structure.

If the question is now raised as to how this contraction of the single protein particles is associated with the splitting of ATP, I can only give some hints. We have seen that both the splitting of ATP by the contractile protein and the contraction of these proteins occur only when magnesium ions are present and when the SH groups of the contractile proteins are not blocked. On the other hand, we have been taught by the chemistry of metabolites that the transphosphorylation of energy-rich phosphate bonds requires magnesium ions in any case and SH groups in most cases. In addition, it is known that hereby the energy is transferred from the phosphate donor to the phosphate receptor. In our case the donor both of energy and phosphate is ATP or ITP. It is the contractile protein which receives the energy and transforms it into work. The indispensability both of magnesium ions and SH groups suggests that also in our case the energy is transferred to the protein by transphosphorylation. This energy is converted into mechanical energy by a series of reactions which changes the energy-rich protein phosphate bonds into low-energy phosphate bonds. After the energy content is exhausted the fragments of the former ATP or ITP are given off. Thus the over-all process has the aspect of a simple breakdown of ATP. These last suggestions are perhaps a little speculative. Such speculations can probably be tested and we shall try to do so.

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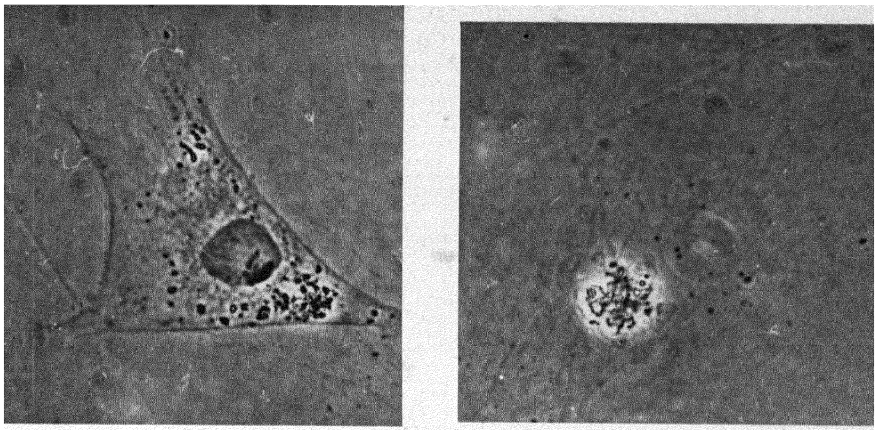
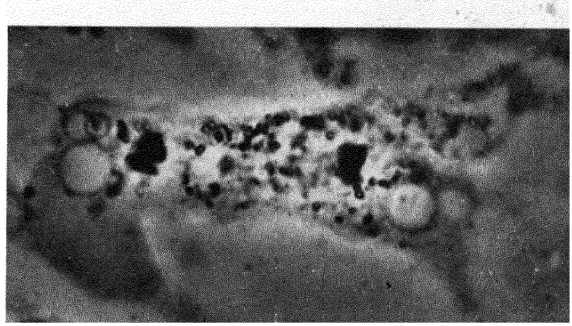


(*a*) (*b*)

Fig. 1.

(*a*)



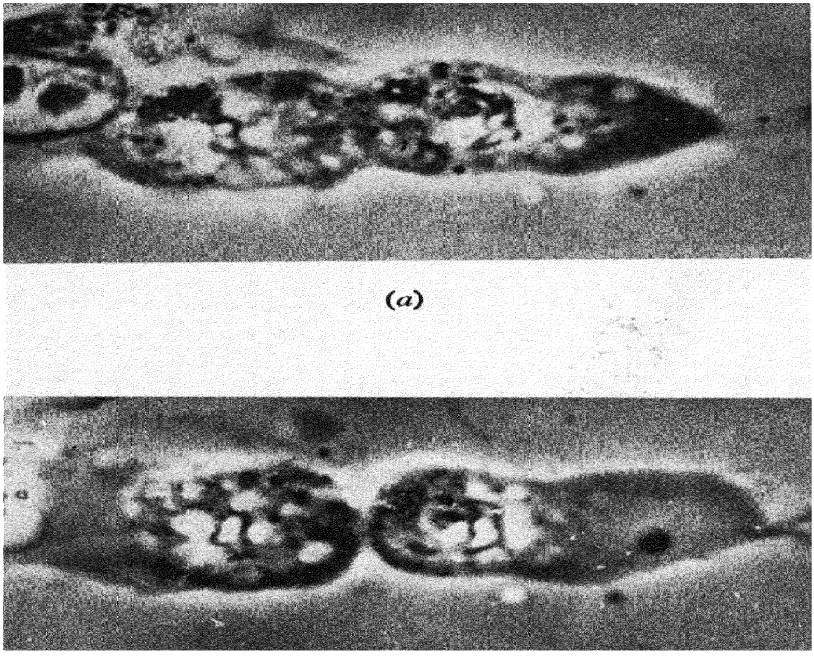
(*b*)

Fig. 2.

For explanation see p. 281

(*Facing p.* 280)

PLATE 2



(*a*)

(*b*)

Fig. 3.

For explanation see p. 281

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Contraction of cell models (amnion fibroblasts) by 2×10^{-3} M-ATP at pH 7·0, ionic strength = 0·14 μ at 37° C. (Hoffmann-Berling, 1954*a*). 28-day extraction. (*a*) without ATP; (*b*) 12 min. after addition of ATP.

Fig. 2. Anaphase movements of cell models (subcutis fibroblasts) by $2{\cdot}5 \times 10^{-3}$ M-ATP at pH 7·4, ionic strength = 0·22 μ, addition of 0·8 M-urea, at 22° C. (Hoffmann-Berling, 1954*b*).

PLATE 2

Fig. 3. Cytokinesis during telophase (subcutis fibroblasts) by $2{\cdot}5 \times 10^{-3}$ M-ATP at pH 7·4, ionic strength = 0·12 μ at 22° C. (Hoffmann-Berling, 1954*d*). Two-hour extraction. (*a*) without ATP; (*b*) 12 min. after addition of ATP.

THE STRUCTURE OF BACTERIAL FLAGELLA

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INTRODUCTORY

Bacterial flagella must be among the smallest organs of motility possible, embodying some mechanism of biological movement stripped to its barest essentials. From this viewpoint it has of course long been desirable to be able to study them at the molecular level, but the need appeared even more urgent after the discovery by X-ray diffraction of the fundamental types of protein fibre structures and their relation to muscular activity (see, for example, Astbury, 1947*a*). It came to be expected then that bacterial flagella would probably be found to belong to one of two main families that we have called the collagen group and the keratin-myosin-epidermin-fibrinogen group, with the odds obviously in favour of the latter because of the power of rhythmic change of form that is at issue and of the characteristic intramolecular elastic properties of the group that could very well underlie it. Little or no reasonable prospect of testing this likelihood presented itself, however, till Gard (1944), using a strain of *Salmonella paratyphi* B, demonstrated the practicability of obtaining adequate yields of highly purified flagella by a procedure based simply on shaking the bacterial suspension for an hour or so in a shaking machine. This detached the flagella cleanly from the bacterial bodies, which were then removed by low-power centrifugation, leaving the supernatant flagella to be dealt with by ultra-centrifugation. Gard finally obtained pellets and viscous solutions of flagella behaving to all intents and purposes like preparations of long thin macromolecules.

It was thereupon decided to undertake a series of more detailed chemical and physico-chemical investigations, and under this scheme data had already been published by Weibull & Tiselius (1945) on the flagellar material prepared by Gard from *S. paratyphi* B, and by Weibull (1948) on the flagella of the harmless and easily cultivated *Proteus vulgaris*, when the collaborative study reported on here was commenced by a visit of Weibull to Leeds with preparations for X-ray examination. Some of the

results of these preliminary experiments, which showed at once that certain bacterial flagella do indeed belong to the k-m-e-f group as had been hoped, were announced shortly afterwards (Astbury & Weibull, 1949), but much more work has since been carried out, with interchange of materials and information, both at Leeds and Uppsala. The present paper describes this work in full and discusses some of its implications.

MAINLY EXPERIMENTAL

We have so far examined the flagella of *Proteus vulgaris* and *Bacillus subtilis*, prepared in the manner just mentioned (Weibull, 1948). The final product from *Proteus*, for example, forms a colourless stable viscous solution with strong flow birefringence. It gives a precipitation reaction with a *Proteus* X19H rabbit antiserum, has a nitrogen content of 15·7–16·1%, shows only traces of phosphorus and 1% carbohydrate at the most, and contains no tryptophane and no purine or pyrimidine bases; it has the characteristics of a protein but not of a nucleoprotein. When a drop of dilute solution is dried down and looked at in the electron microscope, essentially only one structural element is seen—long wavy filaments of the same uniform thickness as the flagella on the bacterium, though generally shorter through some inevitable breakage during the purification process.

The flagella are also reversibly precipitable with ammonium sulphate and show electrophoretic mobility; in brief, they are so obviously of the nature of long macromolecules such as myosin, sodium thymonucleate, gelatin and many others that they invite X-ray study by techniques now sufficiently familiar to be followed almost automatically. Chiefly, we have made very thin films by allowing small pools of the viscous solution to dry down on microscope slides previously dipped in dichloro-dimethyl-silane, washed with water and dried in order to make them water-repellent. This pre-treatment was in a sense the most important detail of the whole investigation, for certainly without it we could never have detached the extremely fragile films from the glass without disintegrating them and so ruining the preferential orientation that is required for X-ray diagrams of any value. As it was, they came away quite easily, and specimens suitable for X-ray examination were built up from several strips lying flat on top of one another.

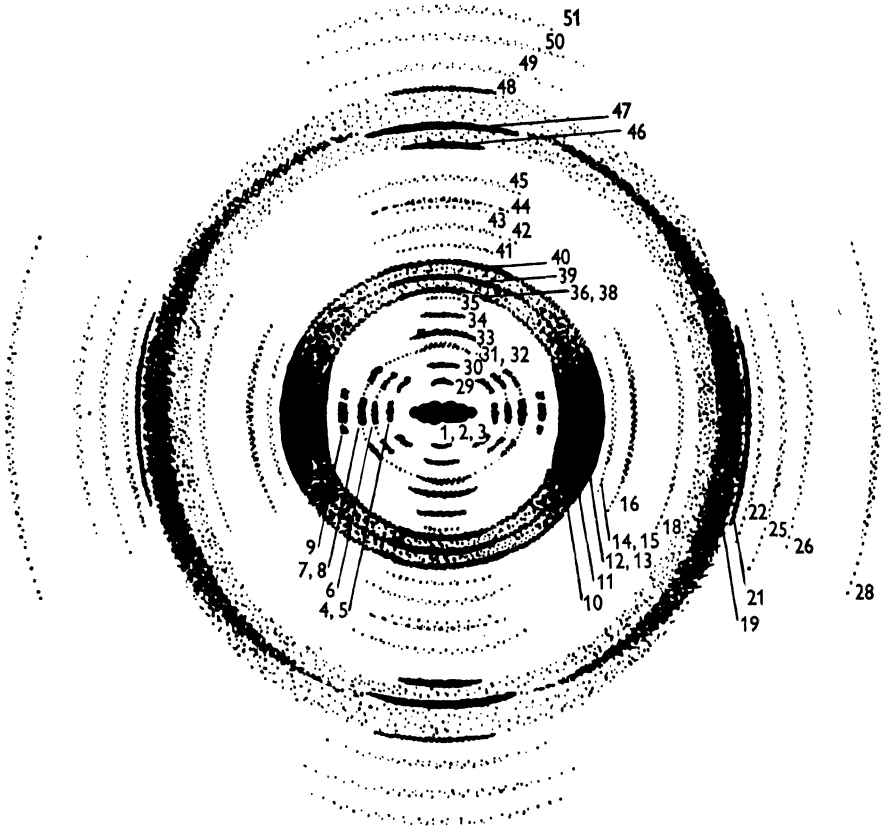
It should be noticed that the drying process causes no significant deterioration; provided the films were not kept too long they could usually be redissolved in water to give solutions still showing flow birefringence.

The object of making thin films by evaporating pools of sol was of course to bring the flagella to lie down as nearly as possible parallel to one plane.

With myosin, for instance (Astbury & Dickinson, 1940), this step was preliminary to cutting narrow ribbons which could then be stretched, a procedure for producing fibre orientation that is often more effective (especially if the stretched ribbons are photographed with the X-ray beam parallel to their plane) than one based on squirting the solution through a fine tube into a precipitating bath. Ribbons of flagellar film, however, could not be stretched by more than a small percentage without breaking, though a number of gentle devices were tried, and no marked improvement in orientation was achieved in this way. Another method (Weibull, 1950*a*) of making fibres by rolling a thick flagellar paste between ground-glass plates was thought at first to give something more than the film technique, but it did not turn out to be so in comparison with results obtained with later films. However, what this approach did give, when a flagellar fibre so produced was photographed in a thin-walled glass capillary sealed with water, was the first demonstration of the great sharpening and increased resolution that occurs in the reflexions from specimens at higher humidities. In the end, most of the X-ray observations reported here were made on unstretched films, photographed with the beam parallel to their plane. This gives the next best thing to a fibre diagram, as will be seen, for example, from Pl. 1, figs. 1 and 2. Altogether, photographs were taken (with Cu $K\alpha$ rays), at distances from about 3 cm. to about 10 cm., of specimens at room humidity or in atmospheres near 0 and 100% relative humidity. Fig. 1 is typical of the best orientation and resolution obtainable at room humidity, while fig. 2 shows the effect of raising the humidity to near saturation. Though the sharpness and resolution is better in the latter, the orientation and overlapping is worse because of the great affinity of the flagellar material for water and the consequent swelling which disturbs the flattening of the filaments brought about by drying down to a thin film. In the ordinary way this trouble would have been overcome by stretching ribbons of film, but for the reason discussed below (which was also the reason why the orientation even in diagrams such as fig. 1 inevitably fell short of what might be expected) that was not feasible, as already mentioned.

Before setting out in detail all the observed X-ray reflexions it will be helpful first to describe the diagrams in brief general terms. The presence on the meridian of the now very familiar reflexion at about 5·1 Å. points at once, of course, to the k-m-e-f group and the α-configuration of folded polypeptide chains, but, besides that, the pattern as a whole is characteristic and especially recalls the diffraction diagrams given by porcupine quill tip and skeletal muscle (see, for example, MacArthur, 1943). These latter are purely α-type diagrams so far as is known, but the flagellar diagrams always show a striking additional feature; the typical α-reflexion at 5·1 Å.

is invariably accompanied by the most typical of β-reflexions, the so-called backbone reflexion at 4·65 Å.; and this is not on the equator as when the α-β transformation is brought about by direct stretching, *but is also on the meridian*, i.e. in the place where it is found with supercontracted specimens of the k-m-e-f group (see Astbury, 1947*a*; and Rudall, 1946, 1950).



Text-fig. 1. Composite fibre diagram of X-ray reflexions from *Proteus vulgaris* and *Bacillus subtilis*.

X-rays, therefore, reveal the simultaneous presence in these flagellar preparations of *two* k-m-e-f configurations, the α and the supercontracted.

Because of the difficulty of reproducing complex X-ray diagrams satisfactorily and the fair number of flagellar photographs now available in any case, we have constructed the composite diagram shown in Text-fig. 1, in which practically all the reflexions observed with *Proteus* and *Bacillus subtilis* are collected together and numbered to correspond with Tables 1 and 2.

Table 1. *Equatorial or near-equatorial reflexions*

	Proteus (~95 % R.H.) Spacing (Å.) and description	*Proteus* (room humidity and drier) Spacing (Å.) and description	*B. subtilis* (room humidity) Spacing (Å.) and description
(1)	—	~100 *vvs* (*j*)	—
(2)	~60 *vvs*	~60 *vvs*	—
(3)	~40 *vs*	~40 *vs*	—
(4)	28 *s* (*a*)	(*k*)	~27½ *s* (*a*)
(5)		23·4 *s*	—
(6)	20·5 *ms* (*a*)	19·6 *w* (*l*)	~22½ *w* (*a*)
(7)	—	15·35 *s*	—
(8)	14·8 *s* (*p*)	—	14·8 *s* (*c*)
(9)	12·6 *ms* (*a*)	12·2 *ms*	12·5 *ms* (*a*)
(10)	10·7 *vs* (*c*, *d*)	10·3 *vs* (*m*)	10·7 *vs* (*c*)
(11)	9·3 *s* (*a*)	8·9 *vs* (*m*)	9·3 *vs* (*a*)
(12)	8·25 *s*+ (*c*, *d*)		8·15 *vs* (*c*)
(13)	—	7·8 *vs* (*m*)	—
(14)	7·5 *ms* (*b*)	—	7·4 *vw* (*b*)
(15)	—	~7 *w*	—
(16)	6·6 *ms* (*b*)	—	6·5 *ms* (*b*)
(17)	—	—	~5·8 *w* (*b*)
(18)	5·3 *w* (*b*, *f*)	—	~5·3 *w* (*b*)
(19)	4·75 *ms* (*c*, *g*)	—	—
(20)	—	—	4·65 *ms* (*c*, *n*)
(21)	~4·2 *ms* (*c*, *h*)	~ 4·2 *s*	4·2 *ms* (*c*)
(22)	4·03 *w* (*b*, *e*)	—	—
(23)	—	—	3·91 *w* (*c*, *o*)
(24)	3·86 *w* (*i*)	—	—
(25)	3·63 *mw*	—	—
(26)	3·43 *mw*	—	—
(27)	—	—	3·27 *w* (*c*, *o*)
(28)	—	~ 3·1 *mw*	—

Notes. *vvs*, exceptionally strong; *vs*, very strong; *s*, strong; *ms*, moderately strong; *mw*, moderately weak; *w*, weak; *vw*, very weak; *a*, definitely equatorial; *b*, probably equatorial; *c*, arc; *d*, rather thick for a single line; *e*, fuzzy; *f*, very fuzzy and perhaps double; *g*, perhaps part of continuous ring corresponding to backbone reflexion of β-configuration; *h*, perhaps continuation of meridional arc; *i*, vague; *j*, includes measurements from 10 cm. photos; *k*, appears to correspond to a non-axial reflexion at roughly 30 Å.; *l*, appears to correspond to a non-axial reflexion at roughly 20 Å.; *m*, strong group of three; *n*, perhaps backbone reflexion of β-configuration; *o*, possibly equatorial; *p*, outer component of probably a close pair of arcs.

It will be convenient first to consider the meridional or near-meridional reflexions. As would be expected from the behaviour of other biological and synthetic fibres, there are no significant variations in the spacings of these reflexions on changing the water content, and on the basis of the final best values listed in Table 3 (from which, for reasons which will be expanded below, the characteristic k-m-e-f 'large-angle' reflexion at 5·1 Å. and the β reflexion at 4·65 Å. have been omitted) the next step is to try to arrive at an estimate of the full crystallographic period along the fibre axis —this is approximately 198 Å. for α-keratin, for example (MacArthur, 1943; Bear, 1943).

Table 2. *Meridional or near-meridional reflexions*

	Proteus (~95 % R.H.) Spacing (Å.) and description	*Proteus* (room humidity and drier) Spacing (Å.) and description	*B. subtilis* (room humidity) Spacing (Å.) and description
(29)	37·3 *w* (*a*, *c*)	—	—
(30)	25·8 *ms* (*a*, *d*)	25·2 *ms*	25·7 *ms* (*b*, *h*)
(31)	19·6 *vw*	—	—
(32)	—	18·5 *vw*	—
(33)	16·0 *ms* (*b*)	15·8 *ms*	16·0 *ms* (*h*)
(34)	12·8 *ms* (*a*, *e*)	12·8 *ms*	12·5 *w* (*a*)
(35)	~11·2 *vvw*	—	—
(36)	—	10·8 *s*	—
(37)	—	—	~10½ *s* (*h*)
(38)	10·15 *w*	—	—
(39)	~9·3 *vw* (*f*)	9·3 *ms*	—
(40)	8·54 *ms*	8·6 *ms*	8·43 *s* (*h*)
(41)	~7·9 *vvw*	—	—
(42)	7·28 *mw* (*a*)	7·26 *vw*	7·34 *vw* (*b*, *h*)
(43)	6·4 *mw* (*g*)	—	6·45 *w* (*b*, *h*)
(44)	6·2 *mw* (*a*)	6·26 *vw*	(*b*, *h*)
(45)	5·65 *vw* (*a*)	5·7 *w*	5·72 *vw* (*b*, *h*)
(46)	5·13 *s* (*)	5·11 *s*	5·11 *s* (*a*)
(47)	4·65 *vs* (**)	4·65 *s*	4·65 *s* (*a*)
(48)	4·25 *s* (*a*) (†)	4·23 *mw*	4·3 *ms* (*b*) (†)
(49)	4·0 *w* (*g*)	—	3·95 *mw* (*b*, *h*)
(50)	3·7 *vw*	—	3·73 *mw* (*b*, *h*)
(51)	3·45 *vw*	—	3·47 *ms* (*b*, *h*)
(52)	—	—	3·22 *w*
(53)	—	—	2·98 *w*

In addition, there are reflexions lying on near-equatorial layer-lines of which the translations parallel to the fibre axis are approximately 200, 100 and 50 Å.

Notes. *vs*, very strong; *s*, strong; *ms*, moderately strong; *mw*, moderately weak; *w*, weak; *vw*, very weak; *vvw*, extremely weak; *a*, definitely meridional; *b*, probably meridional; *c*, fairly sharp; *d*, sharp; *e*, very sharp; *f*, parts of arcs; *g*, fuzzy; *h*, arc; *, characteristic α-reflexion; **, characteristic β-reflexion, used for calibration; †, corresponds also to a fatty reflexion of varying intensity but probably slightly smaller spacing.

Table 3. *Final best estimates of significant meridional or near-meridional spacings*

(*a*) 37·3 Å.	9·3 Å.
(*a*) 25·6	8·57
19½	7·9
18½	(*a*) 7·27
(*b*) 15·9	6·4
(*a*) 12·8	(*a*) 6·23
11·2	(*a*) 5·7
10·8	(*a*) 4·24 (†)
10·15	

(*a*) = definitely meridional; (*b*) = very probably meridional.

(†) Corresponds also to a fatty reflexion of varying intensity but probably slightly smaller spacing.

The period is not the same as that of α-keratin, though a number near to it, viz. 204½ Å. gives reasonable agreement for all the spacings except 37·3 and 19½ Å. Much therefore turns on the accuracy of the 37·3 Å. measurement. The nearest orders of 204½ Å. are the 5th and 6th at 40·9 and 34·1 Å. respectively, but we have checked very carefully that these values are definitely outside our errors of measurement. The first undoubted coincidence between multiples of the two highest observed spacings, 37·3 and 25·6 Å., is at $11 \times 37{\cdot}3 = 410{\cdot}3$ and $16 \times 25{\cdot}6 = 409{\cdot}6$. Taking the mean as practically 410, the resulting comparison between observed and calculated spacings is shown in Table 4.

Table 4. *Comparison between observed and calculated meridional spacings on the basis of a fibre period of* 410 Å.

Order of 410 Å.	Calc.	Obs.
11	37·3	37·3 (*a*)
16	25·6	25·6 (*a*)
21	19·5	19½
22	18·6	18½
26	15·8	15·9 (*b*)
32	12·8	12·8 (*a*)
37	11·1	11·2
38	10·8	10·8
40	10·25	10·15
44	9·3	9·3
48	8·54	8·57
52	7·88	7·9
56	7·32	7·27 (*a*)
64	6·40	6·4
66	6·21	6·23 (*a*)
72	5·70	5·7 (*a*)
96	4·27	4·24 (*a*) (†)

(*a*) = definitely meridional; (*b*) = very probably meridional.

(†) Corresponds also to a fatty reflexion of varying intensity but probably slightly smaller spacing.

It will be seen that the agreement is excellent. There is an approximate coincidence between multiples of 37·3 and 25·6 at $9 \times 37{\cdot}3 = 335{\cdot}7$ and $13 \times 25{\cdot}6 = 332{\cdot}8$ which gives somewhat inferior but still not unreasonable agreement, and which might cast doubt on whether 410 Å. is really the correct answer, but in view of the additional evidence of layer-line translations at roughly 200, 100 and 50 Å. and the fact that 410 Å. is almost identical with the X-ray fibre period found for skeletal muscle—this is not likely to be another accident—we believe that our inference is justified.

In trying to deduce correctly a fibre period of this magnitude there is of course increasingly less significance in the smaller of the meridional spacings listed above, but in the region where they become smaller still, which may usefully be dealt with now, they take on in this particular

tance an important new significance of a diagnostic character. We refer cially to the outstanding reflexion at about 1·49 Å. first observed by cArthur (1943) in α-keratin diagrams and more recently discussed by ıling & Corey (1951) and by Perutz (1951) in support of the former hors' 3·7-residue helical model for α-structures. We have found this exion again quite clearly with films of *Proteus* flagella, while at the same e satisfying ourselves that it is given also by other well-recognized mbers of the k-m-e-f group besides keratin. One of the best diagrams ey were taken in a cylindrical camera of radius 2·98 cm.) was obtained h an oriented strip of epidermin prepared by Dr K. M. Rudall. It is wn in Pl. 2, fig. 1*a*, with the flagellar diagram (Pl. 2, fig. 1*b*) for comison. The latter is naturally inferior because there is only film orientation pposed to the fibre orientation of the epidermin strip.

n our tests we have not noticed any variation of the spacing outside our sible experimental error: it appears to lie consistently within the range) to 1·50 Å., though mostly nearer to 1·49 Å. To the nearest significant ıre our observed values are: porcupine quill tip, 1·49 Å.; epidermin ıdall), 1·50 Å.; myosin (Bailey), 1·49 Å.; tropomyosin (Bailey), 1·49 Å.; in (Rudall), 1·49 Å.; *Proteus* flagella, 1·49 Å. There were other reflexions ides that at 1·49 Å., but they were not easily measurable except in the cupine quill and epidermin diagrams. Beyond the overwhelmingly verful reflexion at about 5·1 Å., porcupine quill showed $4{\cdot}1_8$ Å. (*s*), Å. (*s*), $3{\cdot}4_0$ Å. (*w–m*), $3{\cdot}0_1$ Å. (*m–s*), $2{\cdot}5_6$ Å. (*w–m*), and $1{\cdot}48_6$ Å. (*m–s*); le epidermin showed $4{\cdot}1_8$ Å. (*m–s*), 3·0 Å. (*m*), 2·2 Å. (*w–m*, diffuse halo), Å. (*m–s*). In both these diagrams the reflexion at $4{\cdot}1_8$ Å. is very bably due to a fatty contaminant.

'his further evidence of the 1·49 Å. reflexion makes it certain that the ellar protein belongs to the k-m-e-f group, but still another convincing ıment is supplied by the α-β transformation that we were able to bring ut by stretching at room temperature—not by direct pulling, to be , but by squeezing moist film between pieces of plate glass, which nded it in all directions. The transformation was incomplete by this hod, nevertheless the new backbone reflexion at 4·65 Å. was quite ıistakable.

good oriented β-diagram was later obtained by means of a valuable ınique developed by Rudall (1950, 1952, 1953), who had found that ps of epidermin and myosin, for example, could be stretched very much her without breaking in hot saturated aqueous solutions of ammonium ohate. With the increased internal cohesion thus imparted the α-β transnation could be carried more or less to completion throughout the ole specimen, and there resulted unusually perfect and well-oriented

β-diagrams. On trying the method with tropomyosin, however, to see whether films of this member of the k-m-e-f group, which had always so far broken at extensions of a few per cent, could also at last be persuaded to stretch directly into the β-form, the surprising observation was made that in this case a well-oriented α-diagram was produced. We thereupon treated the flagellar film similarly, actually with the hope that we might now be able to perfect our α-orientation and so obtain conclusive measurements on the macro-period along the fibre axis, but only to find that the outcome was a β-diagram, as with epidermin and myosin. At temperatures below about 80–85° it was still not possible to stretch the film appreciably, and unfortunately by then it had changed over into a disoriented β-structure in any case; but when the temperature of the saturated ammonium sulphate was raised by another 10° or so, a remarkable strengthening of texture occurred: the film became capable of quite rough handling and could be stretched by some 200% without rupture. The resulting β-fibre diagram is reproduced in Pl. 2, fig. 2; and it should be noticed that the cross β-reflexion, indicative of the supercontracted state on our interpretation, has still not been fully destroyed even by this rather drastic treatment.

We may conveniently consider, too, at this point the meridional reflexion at about 4·2 Å. just beyond the cross β-reflexion. In Pl. 2 it is almost certainly due to a fatty contaminant, and in fact the later films sent from Uppsala all showed this feature in varying strength which could not very well be explained in any other way. It is very doubtful, though, whether that is all it amounts to, for we have failed to remove the reflexion entirely by treating the films with fat solvents, and the spacing of the reflexion which persists (probably 4·24 Å.) is somewhat higher than that of the fatty reflexion normally found in this region. We believe that, overlapping a variable fat reflexion, there is an independent meridional reflexion from the flagellar protein itself.

Turning now to the equatorial reflexions, it has to be admitted that we have had little success so far in correlating them crystallographically. The three intense innermost, at about 100, 60 and 40 Å., arise almost certainly from roughly hexagonal, close-packing of the flagella themselves, taken as approximately 120 Å. thick. This assignment is supported directly by electron micrographs of *Proteus* flagella compared with tobacco mosaic virus particles in the same field. The thickness of the flagella is definitely less than that of the virus particles (150 Å.), and about 120 Å. seems a very reasonable estimate. The argument as to close-packing is substantiated by photomicrographs and electron micrographs of salted-out preparations and films which bring out most impressively how the flagella tend to lie parallel in compact wavy aggregates; when they are highly purified, in fact, they

can be seen to fit together so regularly as to 'crystallize' over limited regions (Weibull, 1950*b*). A beautiful example of a wavy 'lock' of *Proteus* flagella is shown in Pl. 3. It was found on breaking up in water an old film that had been kept too long for complete re-dispersal. Pl. 3 could very well be mistaken for a picture of a lock of hair, and it is symbolic and peculiarly apt that this should be so, seeing that the flagella are of the nature of monomolecular hairs.

In this same context it should also be mentioned now that it was these electron-microscope studies of the structure of flagellar films that finally satisfied us why we never succeeded, and would be unlikely ever to succeed, in obtaining quite as good orientation as we felt we had a right to expect. The persistence of the wave form and the strong tendency to preserve it, too, in close-packed aggregates both sets a limit to the planar orientation that can be attained and prevents afterwards any useful pulling out straight by stretching. It was observed (Weibull, 1950*b*) that treating the flagella with formaldehyde results in their straightening out, but this only raised false hopes again, for X-ray diagrams revealed then that the effect was produced only at the cost of serious denaturation.

If indexing the equatorial reflexions on the basis of hexagonal close-packing of individual flagella be continued to spacings smaller than the three innermost just considered, the next two, for instance, might correspond to $d_{13\bar{4}0} = 29$ Å. and $d_{14\bar{5}0} = 23$ Å.; but it is very doubtful indeed whether such a step could be justified. There seems to be a difference of type—an increased definition and reliability of occurrence—suggesting that these remaining reflexions are not of inter- but of intraflagellar origin. They form a system already broadly familiar from other members of the k-m-e-f group (cf. 27·7 Å. (*s*), 12·2 Å. (*vw*), 11·2 Å. (*s*), 10·4 Å. (*s*), 9·2 Å. (*vs*) and 7·8 Å. (*w*) found by Sikorski (personal communication) with Cotswold wool), and in particular the strong reflexion at about 27 Å. and the grouping in the region of 10 Å. would appear to be characteristic, falling as they do into a pattern known for keratin for many years (Astbury & Street, 1931). The complex detail of this pattern that has been resolved more recently has still not brought any corresponding interpretative enlightenment, and now a similar obscurity covers the equatorial reflexions reported here for bacterial flagella. It is by no means impossible to detect various restricted relationships which might have structural significance: for example, if we take as rectangular axes the 23·4 and 19·6 Å. spacings given by dry *Proteus* flagella, there is rough agreement with calculated spacings at 15·1, 11·7, 10·1, 9·1 and 7·8 Å.; but such correspondences, though not of course to be cast aside till we know better, carry little or no conviction at the moment. Approaches by way of a hexagonal

close-packing of quasi-cylindrical, subflagellar fibrils also quickly run into difficulties.

The sharpening and increased resolution of the equatorial reflexions at higher humidities (Weibull, 1950*a*) is a clear indication, however, of considerable regularity of internal structure given the stabilizing effect of water molecules. The presence of the latter is marked by certain spacing changes too (apparently increases for the most part, though there is some ambiguity in our data so far), but it is striking how comparatively small these changes are in view of the great hygroscopicity of the flagella as a whole. They suggest relatively anhydrous invariant units rather like the protein molecules in wet and dry haemoglobin (Boyes-Watson, Davidson & Perutz, 1947).

Finally, in the problem of designating the equatorial reflexions, we must not overlook the unfailing co-existence in these bacterial flagella of *two* polypeptide chain configurations: there is not only the normal α- but also the β-form, and the latter is, moreover, in the state that we have called 'cross β', with the dominant 4·65 Å. reflexion on the meridian. We might therefore expect to find on the equator, from the cross β-form alone, at least a side-chain reflexion in the neighbourhood of 9·8 Å., and perhaps besides a weak reflexion round about 3·3 Å. corresponding to the period of the amino-acid residues. There are actually reflexions that could meet these requirements, but unfortunately there are no means yet of deciding which, if any, really belong to the β-component of the system.

MAINLY DISCUSSION

There is now a great deal of evidence—from chemical analysis, X-ray diffraction, electron microscopy, and other tests (Weibull, 1950*c*, 1951*a*)—to show that at least 95% of the substance of the purified flagellar preparations that we have been investigating consists of a characteristic protein or protein complex which it would be justifiable henceforth to treat as such and to which it would be appropriate and helpful to give a name. We propose, therefore, to call this new member of the k-m-e-f group 'flagellin'.

On the basis of our first X-ray photographs, before the more detailed analysis set out above, we ventured to describe bacterial flagella as being of the nature of monomolecular hairs or muscles. This description, or rather classification, still holds in the fundamental sense that all the members of the k-m-e-f group are on a par as regards their molecular plan and principal elastic properties, but it seems permissible now to go further and claim that, more precisely, bacterial flagella are effectively monomolecular *muscles*.

The most plausible value of the macro-period along the fibre axis is about 410 Å. After reaching this conclusion we received a communication from Prof. Richard S. Bear, of the Department of Biology of the Massachusetts Institute of Technology, kindly supplying for comparison his own latest and best data on the macro-period along skeletal muscle. From an X-ray study of isolated fibrous actin it had already been inferred (Astbury, 1947*b*, 1949) that the higher meridional spacings given by, say, the dried sartorius muscle of a frog correspond in some way to the formation of the actomyosin complex which, following the work of Straub and Szent-Györgyi, has come to be accepted as the true seat of the contractile properties of muscle—that, in fact, the muscle diagram amounts to the sum of

Table 5. *The X-ray fibre period of skeletal muscle (Bear)*

Measured spacings		Fibril period	
Actin (Astbury) Å.	'Type II' fibrils	Layer-line index, *k*	(*k* times spacing) Å.
54	58·2	7	407
	50·8	8	406
26·8	27·2	15	408
18·1	18·7	22	411
	17·8	23	410
13·5	13·8	30	414
10·9	11·2	37	414
	10·8	38	410
9·1	9·2	45	412
7·9	—	52, 53	415
6·8	6·9	60	411
			Mean 411

the actin and myosin diagrams separately*; but unfortunately it was not found possible to obtain a sufficiently perfect actin diagram to settle the full axial period, beyond saying that it was a little over 50 Å. or a multiple thereof. Bear's results demonstrate that the correct multiple is 8, as shown in Table 5. (Note that in this table 'Type II' fibrils was Bear's provisional name for what are now taken to be fibrils of actomyosin, 'Type I' being paramyosin.) The agreement is very good allowing for the difficulty of resolving the close doublets in the actin diagram, and it may now be regarded as established not only that the macro-period along the actomyosin filaments in dried skeletal muscle is approximately 410 Å., but also that this period is closely associated with the presence of the actin component,

* Isolated myosin gives the meridional reflexions at 5·1 and 1·49 Å. common to all the members of the k-m-e-f group, but not those of higher spacing listed in Table 5, while isolated actin gives the latter but nothing at 5·1 Å. The full muscle diagram shows both sets of reflexions.

though whether by actual combination or simply by parallel alignment with the myosin component is not yet apparent. A similar axial period (about 420 Å.) has since been found by Huxley (1953*a*) in X-ray diagrams of living muscle.

The X-ray findings are substantiated too by what is seen in the electron microscope: pictures of toad myofibrils obtained by Draper & Hodge (1949*a*, *b*) reveal, superimposed on the familiar broader bands, a new set of fine cross-striations spaced at roughly 400 Å.; and there is now also the very recent discovery by Philpott & A. G. Szent-Györgyi (1954) that 'light meromyosin', one of the components of myosin released by tryptic digestion, shows exceptionally beautiful cross-striations of period about 420 Å.

The axial period deduced from the X-ray diagrams of bacterial flagella is not, it must be granted, so well founded as the muscle period; moreover, the observed meridional reflexions in the two cases are not the same orders of 410 Å., but it would be asking too much not to seize on such a close agreement (most improbable by mere chance) and postulate that the flagella are of the type of monomolecular muscles. That the X-ray orders of reflexion of the macro-period are not the same is of little significance within the plan of the k-m-e-f group as a whole and in the light of the differences in chemical constitution between flagellin and actomyosin, e.g. there is practically no cystine (and cysteine) in the former (Weibull, 1949*a*, 1950*c*); but it is of course very relevant to inquire whether the flagella resemble muscle filaments by betraying their macro-period also in the electron microscope. From time to time we have come across fine cross-striations in electron micrographs of the flagella of various other (unidentified) bacteria, and once we thought we detected something of the kind in *Proteus* flagella, but for the present, in spite of repeated attempts, the answer must be taken as negative. The reason would appear to be—and it would suggest that cross-striations are unlikely ever to be discovered in the flagella under discussion here*—that always two phases, α and supercontracted, with discrepant periods, are present simultaneously. We have described above how persistently the detached flagella retain their waviness, and undoubtedly this is an expression of the same thing: they are broken off and die in the very act of transmitting their wave motion and remain more or less fixed like that—a fortunate circumstance for which we have coined the phrase 'molecular rigor mortis' (Astbury, 1951*a*).

* It is doubtful whether the well-marked periodicity observed by Starr & Williams (1952) along the flagella of a motile diphtheroid has any direct bearing on this point, since the striations they describe are the expression of a triply threaded helical structure, the three components of which are individually of the same order of thickness as *Proteus* flagella.

Judging by the X-ray diagrams, they die in the process of transmitting a rhythmic interchange of configuration between α and supercontracted, which again conforms directly with what we have long argued is intrinsically the way a muscle works (e.g. Astbury, 1947*a*). Supercontraction (i.e. to a length shorter even than that of the already folded α-form) is a property common to all the k-m-e-f group given appropriate conditions, and the inference is that muscular contraction is a manifestation (in myosin) of the self-same property wrapped up in a lot of accessory phenomena. Unfortunately, we have no X-ray evidence for what happens during an actual living twitch, only the results of studies, particularly those of Rudall (1946, 1950), on strongly supercontracted states induced in non-physiological muscle and in oriented laboratory preparations of the k-m-e-f group; but these results combine in providing an X-ray test—at least for well-marked supercontraction—in the replacement of the α-diagram by the so-called cross β-diagram, in which the backbone reflexion at 4·65 Å. is on the meridian instead of in its more usual position on the equator. One interpretation of the effect is to suppose that the polypeptide chains fall into folds sufficiently long to behave as segments of β-form lying transverse to the fibre axis (see, for example, Astbury, 1951*b*), but whether or not this view is correct is beside the point for the moment, the important consideration here being the experimental fact that the appearance of the cross β-pattern is an X-ray sign of the development of supercontraction. This sign is clearly present in all the X-ray diagrams of the flagella of *Proteus* and *B. subtilis* and is our strong incentive for concluding that their structure is normally compounded of protein chains in two different states of folding, the α-configuration and the supercontracted, the rhythmic transmission of shortening and recovery along selected chains resulting then in the propagation along the flagellum of a periodic system of bending movements.

Pl. 4*a*, *b* consists of a pair of X-ray photographs that illustrate at a glance the composite character of the flagellar diagram in relation to the transformations of the k-m-e-f group: a picture given by the flagella of *B. subtilis* (*b*) is compared with one specially prepared for us by Dr Rudall from a partially supercontracted strip of oriented epidermin (*a*). On the meridian in both diagrams will be seen not only the familiar 5·1 Å. reflexion of the α-configuration but also the cross β-reflexion at 4·65 Å. The resemblance between the two diffraction patterns is striking.

The idea that bending movements are produced by chain-shortening from the α to the supercontracted configuration is not, however, for the present susceptible of more precise quantitative elaboration in terms of what is observed with laboratory preparations of the k-m-e-f group, the

cross β-diagrams of which may be associated with large overall supercontractions up to the order of half the length of the α-configuration (see also Mercer, 1948, and Sikorski, 1953). The principal wave form assumed by the flagella is probably helical and of pitch characteristic of the organism (see below), but in electron micrographs, e.g. Pl. 5, it appears flattened into something like a sine curve—say pairs of semicircles for the purposes of calculation. Taking the thickness of *Proteus* flagella to be about 120 Å. and the wave-length 2μ, the concave sides of the wave are therefore roughly $\frac{120}{5000}$, i.e. only about $2\frac{1}{2}$ per cent shorter than the convex sides, which seems out of all proportion to artificially induced supercontraction. This difficulty, though, is probably not as serious as it looks, since it is not necessary to assume uniform supercontraction all along the chains; rather is it to be expected, by analogy with muscular activity, that there are pulses or 'pockets' of supercontraction following behind one another where the localized shortening is much greater than the average inferred from the flagellum as a whole.

The transmission of pulses of shortening and recovery along the polypeptide structure of the flagellum presupposes, too, a source where the effect originates and whence, once initiated, it sets out as a chain reaction proceeding automatically from one end of the flagellum to the other. In this regard there is no trouble so far, at any rate in principle. Obviously the composition of the solution in which the flagellum operates plays some part in the final result, but it is highly unlikely to be the prime mover, so to say, in the continuous rapid wave motion that is set up. It is pretty generally accepted now that bacterial flagella are not simply outgrowths of the cell wall, but that each originates in a 'basal granule' (blepharoplast) within the body of the organism. We ourselves have obtained electron micrographs indicating as much, and it has been shown in studies by Houwink & van Iterson (1950), and Bisset & Hale (1951), for example, and most clearly by Grace (1954); while Weibull's (1953) experiments put it beyond doubt that the flagella come from within the cytoplasm. In these experiments the cell wall is dissolved away by controlled treatment with lysozyme, leaving the rest of the cell as an intact structural unit, the protoplast, to which the flagella are seen to be still attached.

Within the last few months it has been reported (Hoffmann-Berling, 1954) that the detached tails of certain spermatozoa, after extraction with water-glycerol solvent, show undulatory movements in ATP solutions, but we know of no evidence that bacterial flagella can perform any of their characteristic movements once they have broken away from the protoplast. What then more reasonable than to invoke the basal granule as the required 'signal box' and periodic source of energy? All things considered, in-

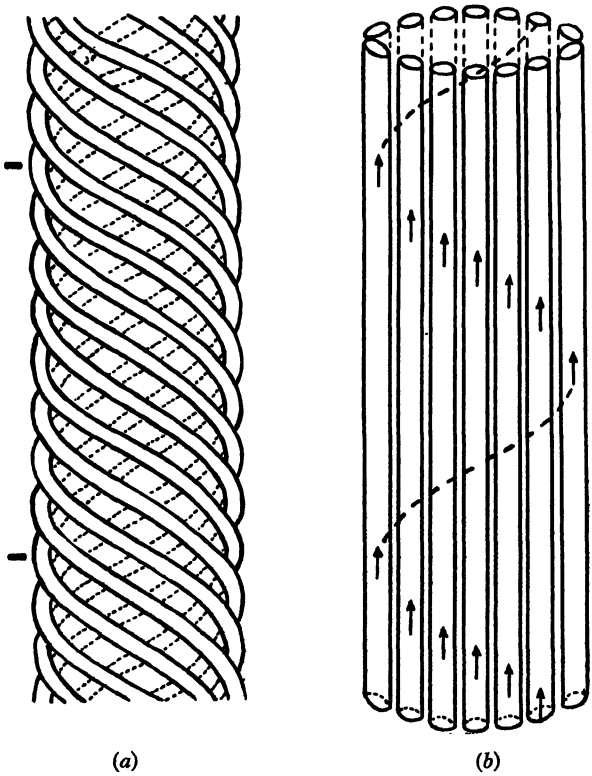
cluding the similar morphological feature in other sorts of flagella besides bacterial, that would seem to be its inevitable function.

To continue now with the problem of the innermost structure of bacterial flagella, and of course in relation especially to their motility, we have to make an attempt at overcoming the double handicap of having for the present neither a detailed analysis of the X-ray diagram nor a direct view of their individual movements when alive. Synchronized beating of groups of flagella ('tails') can be observed under the optical microscope, but single flagella can be resolved only in the electron microscope, by which time they are dead and immobilized. The consistent indications of electron micrographs are, nevertheless, of either a uniplanar wave rather like a sine curve or of a helical wave that has dried down more or less flat; and if we add to this finding the remarkable picture given by the flagella on precipitation with half-saturated ammonium sulphate (Weibull, 1949*b*, 1950*b*, *c*), which shows how they build up left-handed spiral aggregates of constant pitch, practically 2μ for *Proteus* (cf. also Pl. 5) and $2\frac{1}{2}\mu$ for *B. subtilis*, it is a fair conclusion for the purposes of further argument that we must aim, in general, at deriving a molecular mechanism for helical waves. Such a mechanism would then, in any case, include the capability of generating simpler wave forms.

The simplest effect, that of a waving-about motion, could be produced by the supercontraction of polypeptide chains alternately on one side and the other of the flagellum, and from this it is an easy step, depending on the regularity and frequency with which the opposing pulses follow one another and their speed of propagation, first to such movements as the 'breast-stroke' performed by biflagellate algae (e.g. *Chlamydomonas* (cf. Lewin, 1952) and *Polytoma*), and thence to a continuous plane wave train. Schematically, a system of this kind can be represented by two parallel subfibrils and may be likened in cross-section to a double-contact switch 'triggering off' a contractile pulse along each subunit in turn, which is all that is necessary in principle to generate a continuous variety of movements in one plane, and in the limit a train of undulations travelling outwards from the body of the bacterium.

The general case would be a cylindrical array of subfibrils, corresponding in cross-section to a multiple-contact switch. A contractile pulse sets out again along each member of the ring in turn, and the result is now a helical wave train. The basic formal requirement for helical undulations, pointed out by Reichert (1909), is that there shall be a helical 'line of contraction' which is being continuously displaced along the length of the flagellum. One way of achieving this, discussed by Weibull (1951*b*), is for the subfibrils themselves to be all wound spirally as in Text-fig. 2*a*, and for each,

one after the other, to contract and relax *as a whole*. A second method (Text-fig. 2*b*) is to have straight subfibrils not contracting along their whole lengths but only where the appropriate pulse has reached as it travels along each in turn. The first concept is perhaps the more difficult, in that it implies effectively instantaneous intramolecular transformation throughout the entire length of a subfibril; or, if to avoid this objection we assume



(*a*) (*b*)

Text-fig. 2. Two modes of transmitting a helical line of contraction along a flagellum: (*a*) helical subfibrils; (*b*) straight subfibrils. For simplicity, the diagrams represent the initial state, and not the actual change of form produced by the passage of the contractile wave.

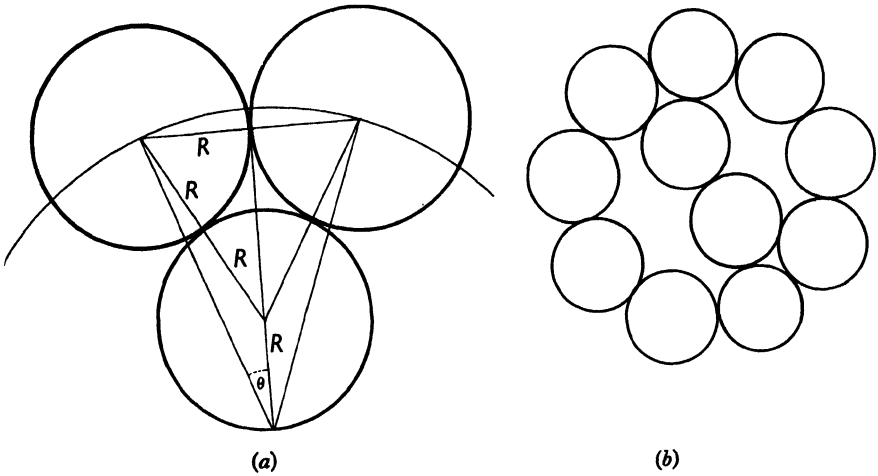
that the shortening is activated from the surrounding medium, it may be asked how, in a flagellum with apparently cylindrical symmetry, the contraction is confined to the line of only one subfibril at a time. Neither view, just yet, can be checked directly at the level of bacterial flagella, where in a thickness of the order of 120 Å. what we have called subfibrils must, however grouped, consist of scarcely more than two or three polypeptide chains, but fortunately it is now possible to look down from an analogous higher level, that of algal flagella, sperm tails and the like. From there we

are beginning to visualize some of the outlines of the mechanism, as will be appreciated from the following considerations.

Outstanding among these is not merely the similarity in kinds of rhythmic movements, especially helical, between bacterial flagella and very much larger flagella and cilia, which is impressive enough when we remember the 'monomolecular' nature of the former and the minute dimensions which have somehow to accommodate an adequate counterpart of the complexity, and comparatively coarse complexity, of the latter; even more so is the common pattern of main components in the larger organelles that has recently been laid bare. In 1946 Jakus & Hall published an electron micrograph showing very beautifully the eleven separated subfibrils of the cilia of *Paramecium*, thereby substantiating like indications with the sperm tails of numerous animal forms obtained by Schmitt, Hall & Jakus as long ago as 1943, but since then workers in many laboratories, taking advantage in particular of developments in sectioning techniques, have contributed to the uncovering of what looks like a master plan for a most surprising range of structures, plant and animal. Convenient references will be found, for example, in Lewin & Meinhart (1953), Bradfield (1953), and Fawcett & Porter (1954), but briefly the scheme calls for eleven subfibrils (often only nine can be detected, but eleven seems to be the full complement, which may or may not be required for all purposes), made up of nine arranged around a cylinder with two others inside; and these last two appear to differ from the surrounding nine in the ease with which they disintegrate in preparative treatments and are lost.

Our own investigations at Leeds with algal flagella, sperm tails and other motile appendages will be reported more fully elsewhere, but because of the unwisdom or impossibility of pursuing the bacterial problem in isolation we should like now to make preliminary mention of a few of our findings in this larger field and try to use them to throw tentative light on the results presented here specially on bacterial flagella. In the first place there is the question of the curious geometry of the arrangement of subfibrils in algal flagella, etc. Clearly it offers at once a multiple-contact or commutator system for the propagation of a helical wave train (or, within this generalized property, the potentiality of simpler movements or wave forms by employing fewer selected 'switches'), but why always a ring of *nine* subfibrils enclosing two others? The clue must surely lie in Text-fig. 3*a*, which shows three circles in contact and from which it follows that the angle 2θ subtended by the centres of the upper two at the lowest point of the third is given by $\cot\theta=(1+\sqrt{3})=2{\cdot}732$, i.e. $\theta=20°\,6'$. To a close approximation, then, nine circles can be arranged in a ring to enclose two others (Text-fig. 3*b*) in just such a way as to allow the latter, in theory, or

conceptually, to 'roll round' inside. We express it in this fashion because so many transverse flagellar sections in the electron microscope tend to convey the idea of comparative emptiness: the two inner subfibrils, when they are visible at all, seem 'loose', without any relation to the enclosing nine—an appearance that must be an artefact of the staining and sectioning procedure, otherwise we are left in the thoroughly unsatisfying, not to say absurd, position of having this special geometrical ring of nine to no apparent geometrical purpose. The argument is that Text-fig. 3 *a* and *b* do



Text-fig. 3. (*a*) Three circles in contact give $\theta = \cot^{-1}(1+\sqrt{3}) = 20° 6'$; hence (*b*) the close approximation of a ring of nine circles enclosing two others.

really portray the system of subfibrillar contacts in the living undistorted state,* and that implicit in the diagrams is the reason for the degree of 'emptiness' which remains even then.

This last is an important point because on crystallographic grounds we might expect hexagonal close-packing, or something near it. We suggest

* The recently published very beautiful study by Fawcett & Porter (1954) of the fine structure of ciliated epithelia now points quite clearly to such a conclusion. For example, in discussing (p. 226) the cilia of the intestinal (typhlosole) epithelial cells of *Elliptio*, they say: 'The whole structure has a diameter of 200 mμ...the other 9 (i.e. the peripheral filaments) are spaced uniformly at a distance of 500 Å. from center to center. The distance from the center of each filament to the membrane is about one-half of this or 250 Å. while from the peripheral filaments to the closer of the two central filaments is again 500 Å.' And in discussing the structure of cilia in general, they say (p. 252): 'It has been observed for instance that the distance between the centers of adjacent peripheral filaments (500 Å.) is about twice the distance from the filament center to the cilium membrane. Also the distance between adjacent peripheral filaments is approximately the same as that between these filaments and the mid-point of the nearest central filament, except where the cilium is obviously and badly distorted. This suggests then that there is a tendency for the filaments to assume positions equidistant from one another very much as though each filament actively organizes around itself as center, a uniform depth of matrix material. The thickness of this zone would presumably be indicated by the width of the margin between the centers of the peripheral filaments and the ciliary membrane (250 Å).'

that the cylinder of nine subfibrils is not the structurally implausible grouping that would appear from single dried cross-sections, but that it is supported and effectively 'filled' by the two inner subfibrils making contact, not with four only of the outer subfibrils, but with all of them in turn; in other words, the inner pair and outer nine are interrelated helically so as to form a kind of rotary impulse transmitter and perhaps also a 'feed-back' device. Though the original activation is still supposed to arise in the basal granule or corresponding centre, this, we feel, is somehow the method of timing and distributing the contractile pulses.

It will be objected that no helical interrelation has yet been observed in the electron microscope and that, on the contrary, the evidence as regards epithelial cilia (Fawcett & Porter, 1954) is that the two sets of subfibrils 'do not coil about each other but remain separate and parallel and retain the same relation to the cilium over their entire length. They confer on the cilium a bilaterality the plane of which (in *Mya* at least) is oriented normal to the plane of pendular motion.' But this finding, it must be remembered, applies so far only to cilia beating in one plane, and many differences in constructional details have been described between one type of flagellar or ciliary equipment and another, differences which cannot all be fundamental. For the most part they will be adaptations to be sorted out and guarded against. There is some common principle underlying all these present-day appearances, some primary mechanism that has been modified to meet innumerable parallel and secondary requirements, and that is what we are looking for.

We shall not develop this concept or speculate on it further on the present occasion, since our main concern is with bacterial flagella; and in any case additional necessary clues to the structure of the larger flagella ought to be forthcoming sooner or later from serial sections for the electron microscope. Our object is to try to extrapolate to bacterial flagella in order to derive some reasonable model wherewith to continue investigations, particularly as regards the interpretation of the X-ray data. Given such a model, the possibility is opened up of calculating the type of diffraction pattern to be expected, as was done, for example, by Cochran, Crick & Vand (1952) when they showed how to check the diffraction diagram given by synthetic polypeptides against the α-helix proposed by Pauling & Corey (1951, 1953*a*). If the bacterial model is indeed a diminished counterpart of the essential motility apparatus of the larger flagella, then corresponding to the visible subfibrils of the latter there should be subunits whose thickness is a little over one-quarter (theoretically 0·255) of the flagellar thickness, say something of the order of 30 Å. This is a figure which at once calls to mind the long-familiar reflexion at 27–28 Å. on the equator of the α-keratin

diagram and the presumably equivalent reflexion listed in Table 1. Pauling & Corey (1953*b*) have recently brought forward arguments for associating the keratin reflexion with a compound helix built up from seven α-helices, but whether or not that view is along the right lines, it looks as if it might be worth while considering the similar reflexion in the flagellar diagram in relation to possible groupings of polypeptide chains with the function of subfibrils. One of the most intriguing aspects of the whole affair is of course the nature of the side-to-side 'insulation' on such a small scale—or maybe that is a wrong idea altogether and that on the analogy of successive stimulation by helical crossing-over in the (9+2) combination we ought rather to think of lateral *contacts*, which at this level would have to be more or less directly by means of the polypeptide side-chains. Another has to do with how such a good X-ray diagram, crystallographically speaking, arises from a structure only about 120 Å. thick. Most of the diffraction pattern must be of intra-flagellar origin, and it is more surprising even than the case of the rod-like units of tobacco mosaic virus, 150 Å. thick, studied by Bernal & Fankuchen (1941). The fact of the mechanism being down at molecular dimensions has, however, at least the advantage that it eliminates secondary appendages and functions that complicate the problem of the larger flagella: for example, Lewin (1952) and Lewin & Meinhart (1953) describe the mating behaviour of cells of *Chlamydomonas moewusii*, which also indispensably involves their flagella. With bacterial flagella, we may be morally certain that in so small a compass there is no room for anything significantly beyond what is demanded by their power of rhythmic movement.

In a chemical analytical study of the flagella of *Polytoma* and other algae now being undertaken at Leeds by Dr J. Tibbs, assisted by Mr A. Millard with the electron microscope, the exploration of techniques for collection and purification has yielded as one of its first results a glimpse of ordered disintegration and breakdown products so reminiscent of current findings on muscle structure that we can hardly conclude the present paper without some slight reference to it in relation to corresponding signs on the bacterial plane. We have summarized above the interplay of chemical and X-ray evidence on muscle that has served to define so much more clearly the twofold character of the contractile actomyosin complex: how myosin lies in parallel with what is called *F*-actin, the fibrous modification built up by the linear polymerisation of corpuscles of *G*-actin, and how the numerical value of the axial period which this combination gives rise to is found again in the X-ray diagram of bacterial flagella; but we cannot leave it at that, since an obvious corollary is that bacterial flagella too (not to mention keratin and other members of the k-m-e-f group) have a particulate side

to their make-up. Weibull (1948, 1950*c*) found some indications of the kind in the irreversible decomposition of the flagella of *Proteus vulgaris*, *B. subtilis* and *Salmonella paratyphi* B at pH 3–4 to produce rather homogeneous particles of weight about 40,000, and it becomes pertinent now to try to make a little more of this observation. The picture given in the electron microscope by disintegrating flagella of *Polytoma* is essentially particulate in the later stages: first are to be recognised the subfibrils, but within these are soon to be seen filaments which in turn break down into regular rows of uniform particles (Pl. 6), until finally these last dominate the whole field. And not only do the subfibrils appear to be roughly one-quarter the diameter of the complete flagellum, as might be expected from Text-fig. 3*a* and *b*, but also the filaments and particles appear to be roughly one-quarter the diameter of the subfibrils. Whether this means a repetition of the (9+2) geometry down at the level of the subfibrils is not known, but the possibility is there to be borne in mind. The investigation in detail is only at its beginning and the quantitative results quoted here are still only tentative, but all the same the general impression is certainly of a structure whose skeleton outlines derive initially from particles, mostly of diameter about 175 Å., though finer constituents, both particles and filaments, can also be detected. The situation is definitely of a similar class to what has recently been developing with muscle, where the actin component, especially as revealed directly in electron-microscope studies of striated muscle (Huxley, 1953*b*; Hanson & Huxley, 1953, 1954—see also this volume), is assuming greater prominence every day. Almost the first thought, therefore, on looking at electron micrographs of the ordered disintegration of *Polytoma* is to seek for likely connections with actin on one hand and the corpuscular breakdown products of bacterial flagella on the other, and that in fact is one of the directions along which our investigations are now proceeding.

We should like to thank Dr K. M. Rudall for preparations of oriented epidermin and for the X-ray diagram of partially supercontracted epidermin; Dr J. Tibbs and Mr A. Millard for the preparation and electron microscopy of disintegrating *Polytoma*; and Mr Millard especially for all the photographic prints of the X-ray diagrams and electron micrographs illustrated here.

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PLATE I

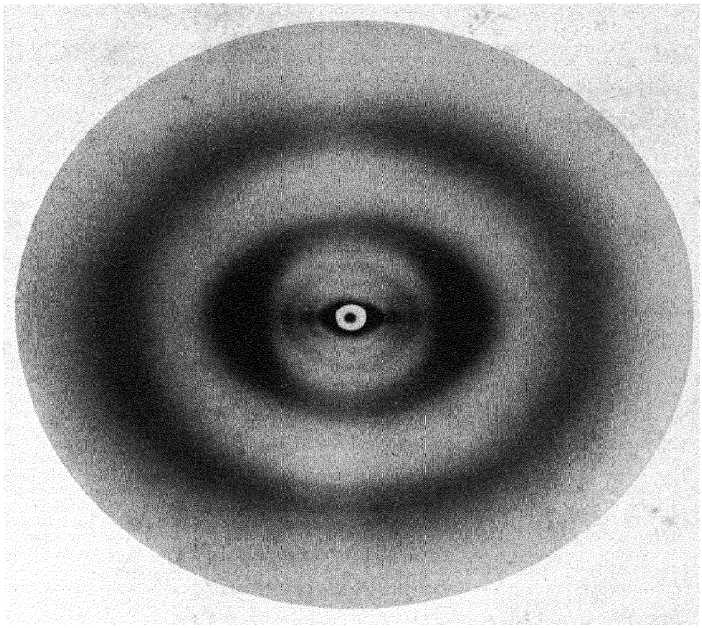


Fig. 1.

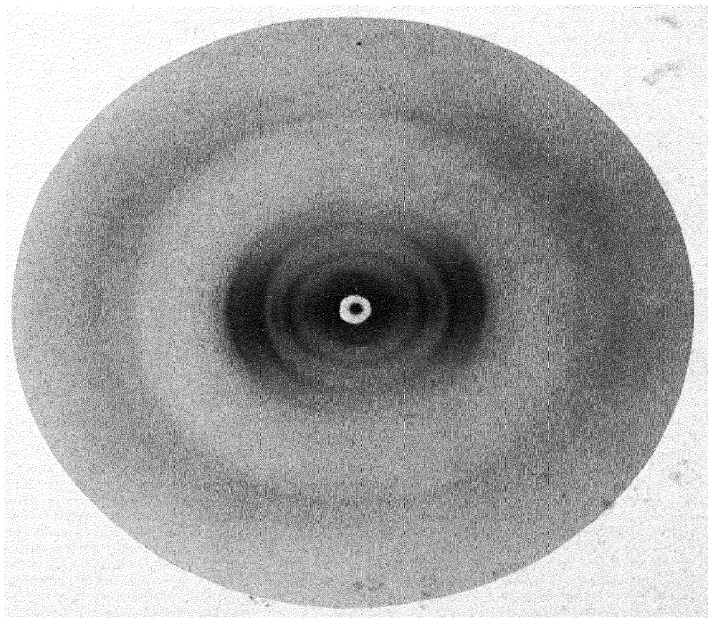
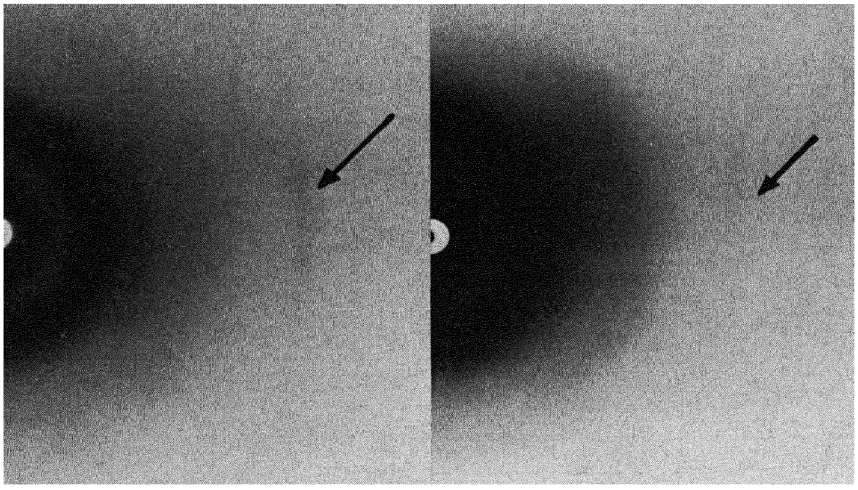


Fig. 2.

For explanation see p. 305



(*a*) Fig. 1. (*b*)

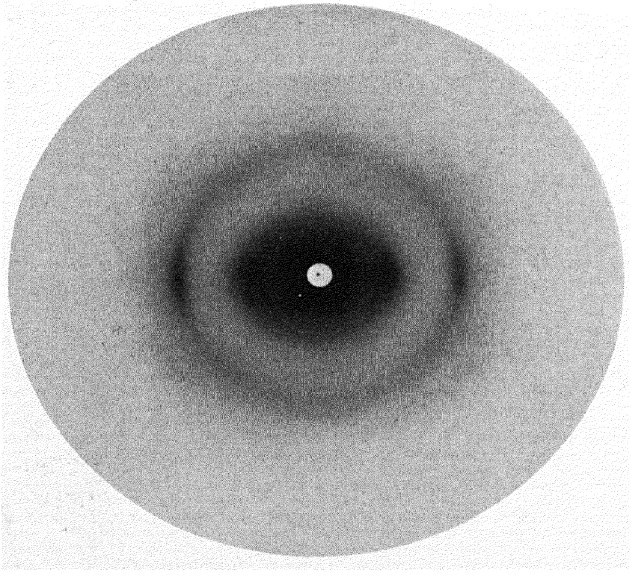
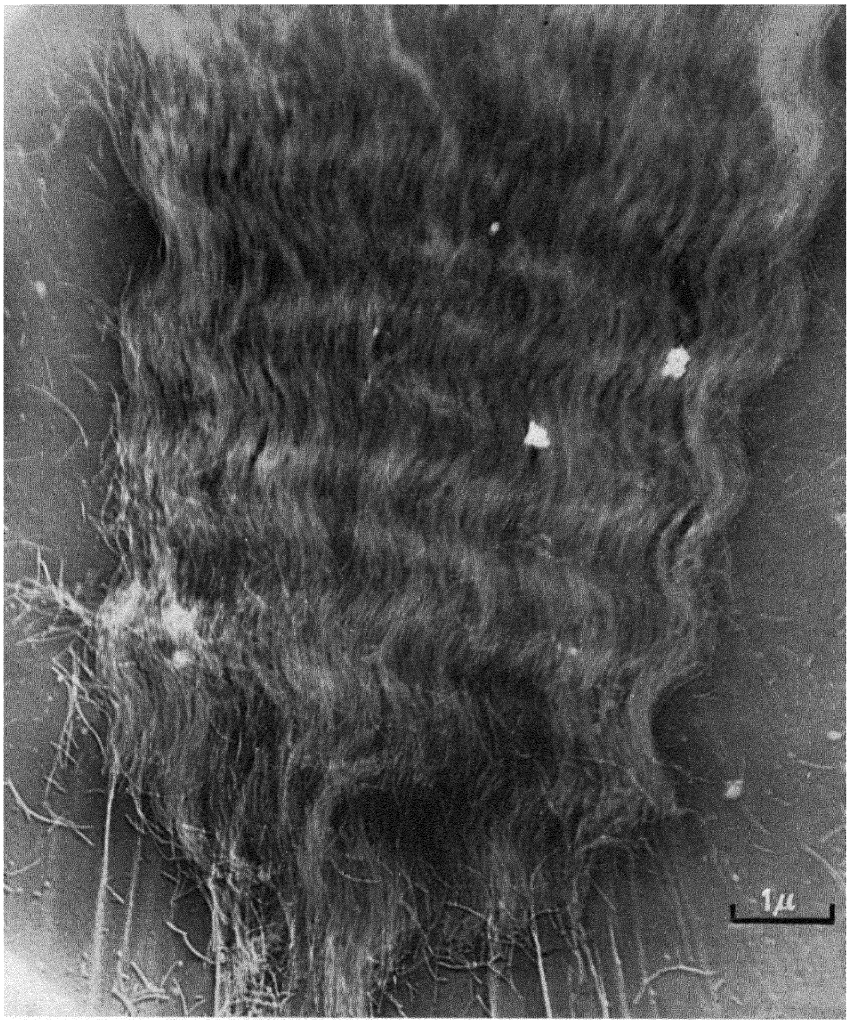


Fig. 2.

For explanation see p. 305

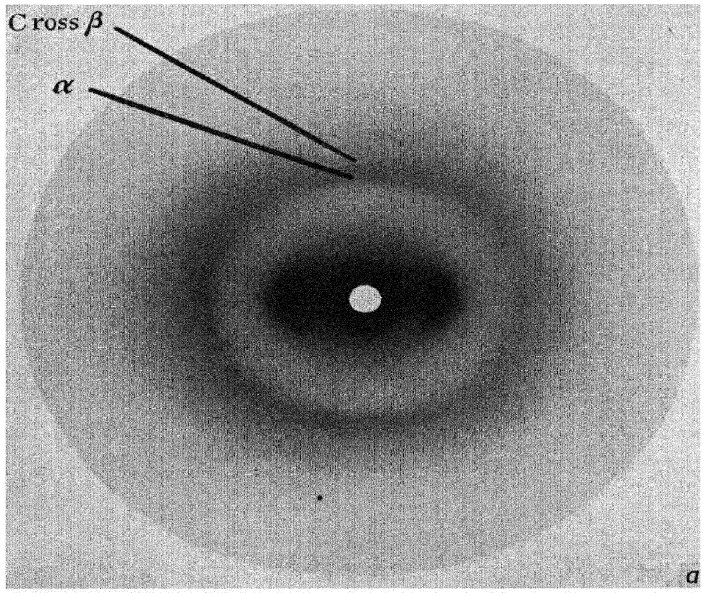
PLATE 3

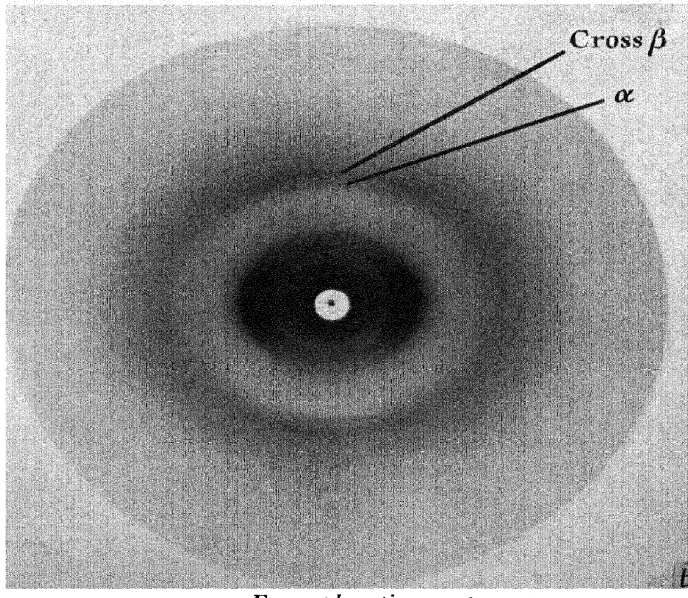


For explanation see p. 305

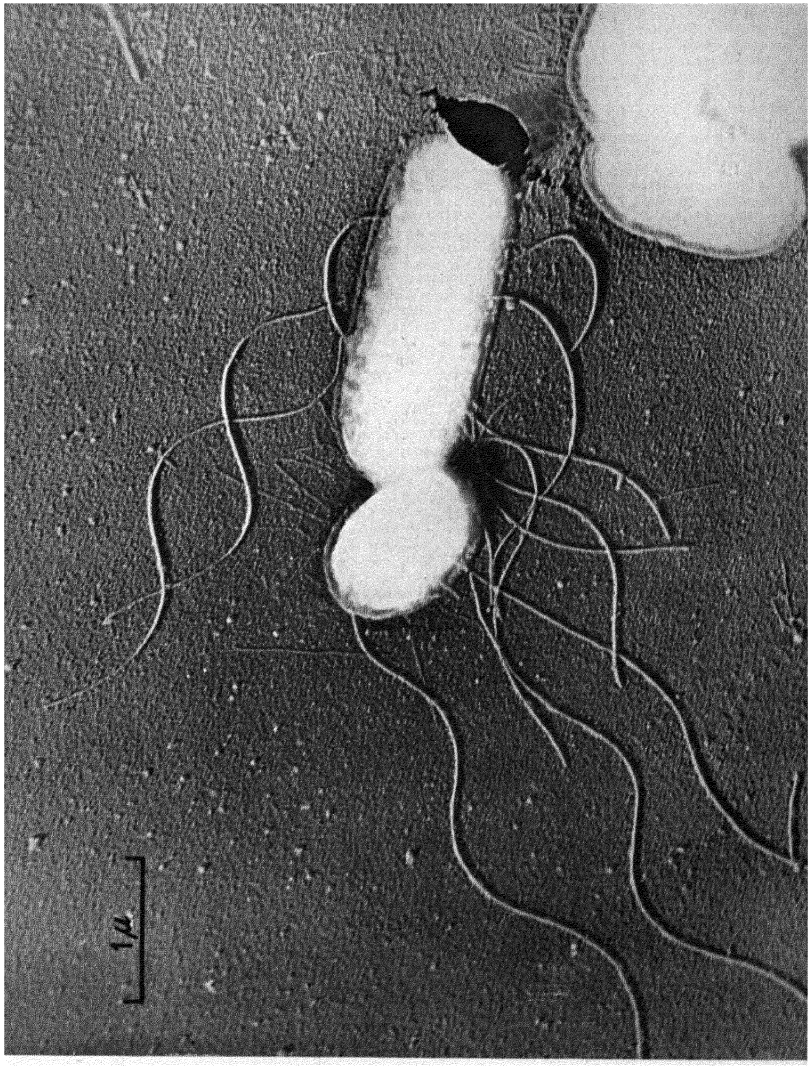
(*Facing p.* 304)

PLATE 4





For explanation see p. 305



For explanation see p. 305

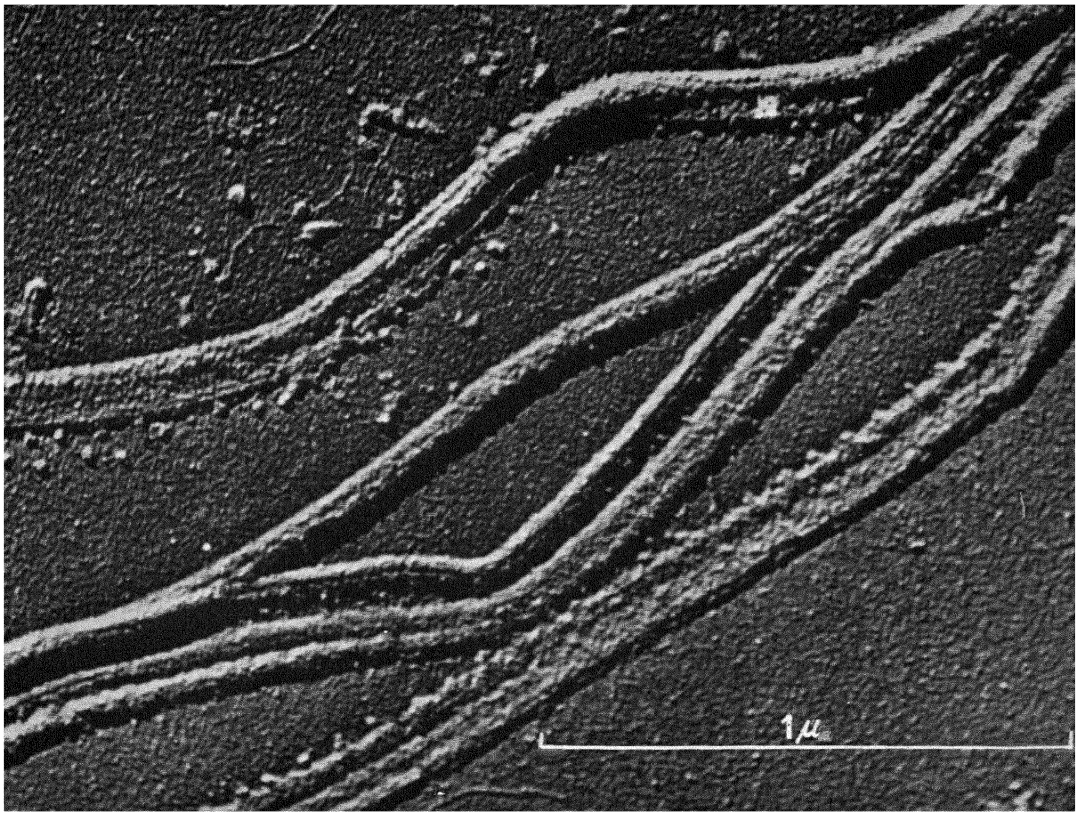


PLATE 6

For explanation see p. 305

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Film of *Proteus* flagella: X-ray diagram taken at room humidity with the beam parallel to the film.

Fig. 2. Film of *Proteus* flagella: X-ray diagram taken at near saturated humidity with the beam parallel to the film.

PLATE 2

Fig. 1. (*a*) X-ray diagram showing the 1·49 Å. meridional reflexion given by an oriented strip of epidermin; (*b*) X-ray diagram showing the 1·49 Å. meridional reflexion given by a film of *Proteus* flagella.

Fig. 2. X-ray β-fibre diagram obtained after stretching a film of *Proteus* flagella by some 200% in hot saturated ammonium sulphate solution.

PLATE 3

Electron micrograph of a 'lock' of *Proteus* flagella.

PLATE 4

X-ray diagram given by a film of *B. subtilis* flagella (*b*) compared with that given by a partially supercontracted strip of oriented epidermin (*a*).

PLATE 5

Electron micrograph showing the wave form of *Proteus* flagella.

PLATE 6

Electron micrograph showing the ordered disintegration of *Polytoma* flagella into subfibrils, filaments and particles.

FIBRE PATTERNS IN ANIMAL FLAGELLA AND CILIA

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Cavendish Laboratory, Cambridge

INTRODUCTION

The view that a cilium or flagellum consists of a bundle of fibres is far from new. For Ballowitz in 1888 described how the tails of many kinds of animal sperm are liable to fray into their component 'elementary fibrils' (each well beyond the theoretical limit of resolution with the light microscope), and in one case—chaffinch sperm—he even figured the precise number of fibres (11) which is now known to occur in so many flagella and cilia. Furthermore, the polarized light studies of Schmidt (1937), as well as of earlier workers, showed in living sperm tails a birefringence in accordance with the presence of longitudinal protein fibres. The pioneers of biological electron microscopy were not surprised therefore to find evidence of longitudinal fibres in cilia and flagella. What is more surprising, in view of the wide variety of functions carried out by these vibratile processes, is that further work with the electron microscope, both on animals and on plants, has revealed the extremely widespread occurrence of a *single* pattern of fibres inside cilia and flagella—a cylinder of 9 peripheral fibres surrounding 2 central fibres, making 11 in all. One of the earliest papers to provide clear evidence of such an arrangement of fibres was that of Grigg & Hodge (1949) on cock sperm. The extensive studies of Manton (1952) and her co-workers have revealed the 9+2 fibre pattern in cilia of green and brown algae, bryophytes and pteridophytes. The elegant recent work of Fawcett & Porter (1954) has done the same for a wide range of animal cilia. The present paper first reviews the structure of animal cilia (incorporating original observations on the cilia of *Mytilus* and *Pleurobrachia*) and of animal sperm tails (with original observations on the sperm of a sea urchin, an ostracod, a frog, and several mammals); it then goes on to discuss (1) what structures constitute the basic essentials for ciliary and flagellar movement, (2) possible ways in which this basic machinery may produce the movements observed, (3) the constancy of the 9+2 pattern and the significance of such departures from it as have been discovered. A more extensive survey and bibliography of early work than can be given here will be found in the paper of Fawcett & Porter (1954).

Nomenclature. For convenience, the terms 'flagellum' and 'cilium' are used throughout in the conventional sense despite the fact that no logical absolute distinction can at present be drawn between them. Certainly no constant structural differences between cilia and flagella have yet been discovered. As regards behaviour, some cilia are known to exhibit a two-dimensional beat with a stiff effective phase and a limp recovery phase (Gray, 1928, 1930), whereas flagella commonly generate a series of waves which are usually approximately two-dimensional, though occasionally corkscrew-shaped; but there are various complications in such a simple distinction and in any case very few examples have been accurately filmed. Finally, as regards distribution, flagella usually occur singly whereas cilia occur together in large numbers, but the more complex flagellates (Polymastigina, Hypermastigina) upset any classification based on this feature.

In describing the internal structure of cilia and flagella, the primary strands (usually 11 in number and 300–1000 Å. in diameter) will be referred to as 'fibres'; the finer strands (75–150 Å. in diameter), of which these primary fibres are composed, will be referred to as 'subfibrils'. It seems probable that these subfibrils will ultimately be resolved into a number of polypeptide chains without any further intermediate levels of organization.

THE STRUCTURE OF ANIMAL CILIA

Mytilus edulis. The general arrangement of cilia on a gill filament of this common marine mussel is indicated very diagrammatically in Text-figs. 1*a* and 1*b*, and was described by Orton (1912) and summarized by Gray (1922) and Carter (1924). Work on the fine structure of gill cilia in other lamellibranchs has recently been reported by Sedar, Beams & Janney (1952) and Worley, Fischbein & Shapiro (1953), as well as Fawcett & Porter (1954). Each laterofrontal cell in *Mytilus* carries two parallel rows of cilia, 20–25 per row (Fig. 10*), with the direction of beat along the row and the effective stroke toward the frontal cilia. Each cilium acts in unison with its partner in the other row, but this seems to be due to precise co-ordination of their beat—perhaps via the rootlets (Bhatia, 1926; Worley, 1941)—rather than to any structure actually uniting the free parts of the two cilia. In some papers, all the cilia of one cell are spoken of collectively as 'the laterofrontal cilium'.

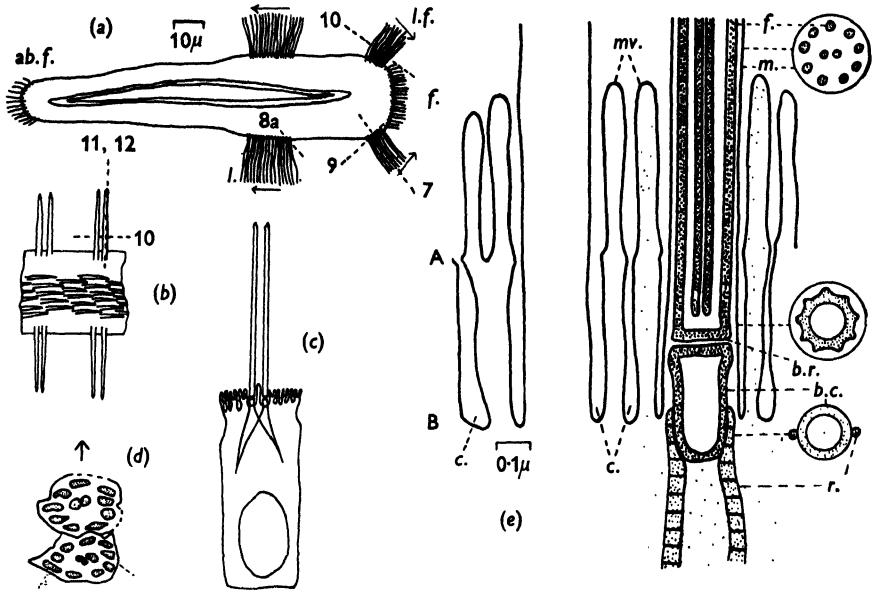
Our observations on the structure of the laterofrontal cilia of *Mytilus* are summarized in Text-fig. 1*e*, which is a section across the two ciliary rows of one cell, showing one cilium from each row. The free part of each cilium (from whatever part of the gill) consists of a bundle of 11 fibres surrounded

* Figs. 7–22 are contained in Plates 1–4.

in matrix and enclosed by a membrane. There are 9 outer fibres, more or less equidistant from each other (440 Å. centre to centre), arranged in a cylinder around two central fibres (Fig. 8). All the gill cilia are about 0·2 μ in external diameter. This is too near the limit of resolution with the light microscope to be checked by direct comparison with wet specimens, but checks of this kind on the thicker flagella of mammalian sperm show that there is very little shrinkage in the external diameter of the tail in sections. Any obvious distortion produced by sectioning can be allowed for by comparing diameters in the direction of cut and at right angles. Nevertheless, it is wise to regard measurements (in sections or frayed, unsectioned tails) on structures as small as individual fibres inside a cilium as giving only a rough estimate of true dimensions. With this caveat, it may be stated that the outer 9 fibres are about 300 Å. in diameter and the 2 centre fibres each about 180 Å. in diameter. Sometimes it appears that the former (not the latter) can be resolved into two subfibrils side by side on the same circumference and each about 200 Å. thick, so that the whole fibre is elliptical, or even dumb-bell-shaped, in cross-section. The micrographs of Fawcett & Porter are superior to ours in this respect, but on the other hand these authors do not point out the difference in the thickness between the outer 9 fibres and the central 2. (In all typical 9+2 cilia and flagella studied in this laboratory, the 2 centre fibres have been little more than half the thickness of the outer 9, so that the *two* centre fibres might be regarded as a loose doublet, equivalent to *one* of the 9 outer doublets. It is possible that the greater tendency of the centre fibres to dissolve (see p. 317) is due solely to their loose pairing, with less binding material and greater surface area, but it seems more probable that the centre fibres themselves are chemically different from the outer 9.) In some of the gill cilia the distal 4–5 μ is only 0·05 μ thick; the arrangement of fibres in these thin tips has not been discovered, and the tips of laterofrontals have not been definitely identified.

At the base of the cilium, the central pair end about 400 Å above the basal plate (apparently without connecting to any other structure) and the 9 outer fibres fuse with each other to form a continuous cylinder which ends on a basal plate or ring. (Or perhaps the 9 fibres become applied to the outer surface of a short cylinder projecting up for 0·4 μ from the basal plate or ring—this would explain better the 'cog-wheel' appearance seen in cross-sections at this level as indicated in Text-fig. 1 *e*.) The basal plate or ring adheres very closely to the basal corpuscle, the gap between them never being more than 250 Å. The basal corpuscle is a hollow cylindrical body about 3200 Å. long and 1800 Å. wide, flat at its outer end and tapering and rounded at its inner end. After osmic fixation its walls are extremely dense to electrons (and impermeable to methacrylate, which

apparently does not penentrate, judging by the very low absorption inside the corpuscle). Closely applied to the outside of the basal corpuscle are two 'ciliary rootlets' about 800 Å thick. The chance serial sections in Fig. 9 suggests that these are not gradual transformations of basal corpuscle, but are distinctly separate fibres. The two roots from each cilium diverge until



Text-fig. 1. Diagrams of *Mytilus* gill cilia. (*a*) Transverse section (T.S.) gill filament showing disposition of frontal (*f.*), laterofrontal (*l.f.*), lateral (*l.*), and abfrontal (*ab.f.*) cilia. Cell details omitted. Cilia somewhat shortened. Arrows indicate the directions of their effective strokes. (*b*) Small part of gill filament viewed from front, i.e. from the position of letter '*f.*' in Text-fig. 1*a*, showing paired laterofrontal cilia, whose effective stroke is vertically up from the plane of the paper, and the frontal cilia drawn at the end of their effective stroke. In both (*a*) and (*b*) the plane of section in various of the electron micrographs is shown by dotted lines labelled with the number of the micrograph. (*c*) Single laterofrontal cell viewed as in (*b*) showing a pair of cilia (one from each row) and their 'cross-over' rootlets. (*d*) Tracing of two cilia from Fig. 10 showing orientation of fibres with respect to direction of effective stroke (latter indicated by arrow). (*e*) Reconstruction of basal regions of one cilium, viewed as in (*c*), showing 9+2 fibres (*f.*), membrane (*m.*), basal plate or ring (*b.r.*), basal corpuscle (*b.c.*), rootlets (*r.*), microvilli (*mv.*) and crypts (*c.*). Cross-striations have not been clearly observed in these rootlets but they almost certainly exist.

the left root from one joins the left root from the other and similarly with the right roots (Text-fig. 1*c*). These roots pass down into the cell through the apical cytoplasm (which is densely packed with mitochondria), but become lost in the nuclear zone.

The ciliary membrane is continuous with the cell membrane just below the basal plate. In fixed preparations the ciliary membrane is usually wrinkled (whether or not the buffered osmic fixative is made up in sea water) as if in the living state, before removal of water, it were distended by

osmotic or other forces. Between the cilia (and over most of the surfa the gill filament) the cell membrane is thrown into small finger-like cesses or microvilli (0·5μ long and 650 Å wide) with crypts of rather sir dimensions at their bases, so that there appear superficially to be two membranes, at *A* and *B* in Text-fig. 1*e*; the ciliary membrane penetrat the inner of these (*B*). The microvilli alone give the outward-facing sur of the cell an area approximately ten times greater than if it were smooth, they must greatly facilitate gaseous exchange.

No connecting links between cilia have been observed other than paired 'cross-over' rootlets described above. Frontal and lateral cili similar to laterofrontals in structure, except that they are not paired an two rootlets possessed by each do not therefore show crossing-over.

Two points remain to be considered: (*a*) the symmetry (if any) of fibre pattern; (*b*) the relation of the fibre pattern to the *direction of beat.* former question is rather complex and will be deferred to a later sec where theories of ciliary movement are considered. As regards the d tion of beat, it is clear now that this is approximately at right angles t line joining the central pair. This can readily be deduced in laterofro (Fig. 10) because the beat direction is along the row. Longitudinal sect through the plane of beat (Fig. 11) confirm this conclusion in that only central fibre can be seen (with occasional exceptions—see below). Wh much harder to deduce is the direction of the *effective stroke*— whethe or down in Text-fig. 5*a*, for example. Such evidence as we have sug that the effective stroke is up in Text-fig. 5*a*, i.e. toward fibre 1. Mi graphs and tracings pointing to this conclusion are shown in Fig. 10 Text-fig. 1*d*, where the direction of the effective stroke is deduced from presence of frontal cilia, toward which the laterofrontal cilia beat. It w be interesting to know the direction of the effective stroke in the exam studied by Fawcett & Porter, who also have found the direction of ci beat to be at right angles to the line joining the central pair. The latter in their diagrams labelled the axis of symmetry; this may be true in but is probably not so, and is certainly not so in mammalian sperm (see below). The fact that the direction of beat in *cilia* is at right angl the line joining the central pair makes interesting comparison with situation in *mammalian sperm* (Text-fig. 3*b* and Fig. 19), where the joining the central pair is at right angles to the diameter on or near w lie the three thickest fibres, and on which one would therefore expec amplitude of the tail waves to be greatest.

It should be mentioned that we have obtained a few micrograph *Mytilus* laterofrontal cilia sectioned longitudinally through the plan beat where, in some cilia, *two* central fibres are visible (Fig. 12). Th

contrary to expectation, for if the beat is at right angles to the line joining the central pair only one central fibre should appear in such sections. It would also mean that the orientation of the central pair varied in different cilia of the same cell, which is contrary to the evidence of transverse sections—though unimportant as regards the theory of ciliary movement advanced below. In some of our longitudinal sections (Fig. 12) there have even appeared two central fibres in one part of a cilium and only one central fibre in another part of the same cilium, as if the central pair were spiralling; but in fact the evidence is fairly clear that the outer 9 regularly, and the central pair usually, run straight down the cilium parallel to its long axis. There are several possible explanations of the apparent discrepancies mentioned above. It may, for example, be that one of the two central fibres seen in the longitudinal sections (Fig. 12) is not a true member of the central pair, but a member of the ring of 9 peripheral fibres artificially displaced toward the centre. Alternatively, it may be that the cilia in Fig. 12 are somewhat disarranged instead of all bending in exactly the same plane as is the case in Fig. 11, where every cilium throughout its length shows only one central fibre, as expected. The remarkable fidelity of preservation which can be achieved is, however, evidenced by Fig. 7, where a transverse metachronal wave, like that described by Gray (1930), is retained in the fixed and sectioned cilia.

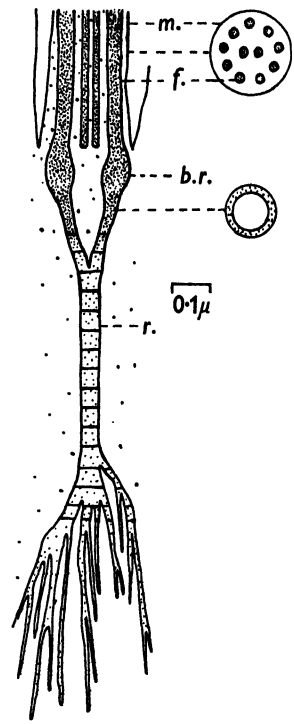
Pleurobrachia pileus. In the comb plates of *Pleurobrachia*, the ctenophore known as 'the sea-gooseberry', cilia are very densely packed, many per cell, and microvilli appear to be absent. The free part of the cilium contains the usual 9+2 fibres, and these are enclosed in a membrane capable of stretching to several times its normal circumference under osmotic forces. At the base the outer 9 fibres join to form a cylinder which bears a transverse thickened ring, but there is not such a large and distinct basal apparatus as in *Mytilus* laterofrontals and there is no division into basal plate and basal corpuscle (Text-fig. 2). Below the basal ring, the hollow cylinder formed by fusion of the 9 fibres is transformed gradually into the single root (again a contrast with *Mytilus*), which is about 400 Å. thick (Fig. 13) and shows a regular periodicity (major period 500 Å.). This single root soon divides into many fine rootlets, which can be traced for at least 2·5 μ into the cytoplasm before they apparently peter out in the nuclear region of the ciliated cell, the general cytoplasm of which is full of these fine fibres. Small, dense, intercellular masses 2–3 μ across are sometimes seen wedged between the cell membranes a few micra from the outer cell surface; their significance is unknown.

In *Pleurobrachia*, but not in *Mytilus*, cilia have been shown to be under the control of impulses arising from a specific sense organ. In neither

organism have nerves been demonstrated in the region of the ciliated cells, but it is hard to believe that they are completely absent. It would be most interesting to re-examine examples such as the velum of a nudibranch larva (Carter, 1926), where nerves apparently come into very close contact with cilia which are under the control of the animal.

Amphibian and mammalian cilia studied by Fawcett & Porter. The two preceding sections summarize observations by the author. To extend this survey of ciliary structure, it is desirable to refer to the interesting work of Fawcett & Porter on amphibian and mammalian cilia. In each case the main part of the cilium resembles *Mytilus* cilia as described above, except that Fawcett & Porter find both in molluscs and vertebrates that each of the 9 outer fibres is clearly double, containing two equal strands lying side by side on the same circumference of the cilium. They found considerable variation in the basal corpuscles and associated structures.

Cilia of the frog's pharynx have basal plates as in *Mytilus*, but their basal corpuscles are curved—very approximately in the form of a 60° arc of a circle of radius 0·5 μ centred on the cell surface. On the convex side of the curved corpuscle, and in the plane of the curve, are two processes—an upper, short, blunt process nearly parallel with the cell surface, and a lower, single, cross-striated root passing into the interior of the cell.



Text-fig. 2. Diagram of basal region of *Pleurobrachia* cilium, showing 9+2 fibres (*f.*), membrane (*m.*), basal ring (*b.r.*), rootlet (*r.*). The latter is cross-striated (period about 500 Å. in sections) and divides into many finer rootlets.

A cilium from mouse oviduct has no sharp break between base and basal corpuscle (as in ctenophores). Just outside the cell the 9 outer fibres join; and the central pair end at the cell membrane. But just inside the cell membrane the 9 fibres are distinguishable again, each hollow and touching its neighbours, and the slightly curved basal corpuscle consists of a tangled mass of these hollow fibres. Rootlets are not found here, but have been reported in other mammalian ciliated cells.

THE STRUCTURE OF ANIMAL SPERM TAILS

So far only a few examples have been studied from the endless variety of types found among animal sperm. As in cilia, so in sperm tails the 9+2 fibre pattern is of extremely widespread occurrence. But whereas no divergences whatever have yet been discovered among cilia, there is at least one quite different pattern found among sperm tails (ostracod sperm) and one considerable modification (mammalian sperm). In general, however, the evolution of animal sperm seems to have involved mainly the rearrangement of the energy-producing mitochondria rather than modification of the pattern of axial fibres. Thus in 'primitive' sperm (sea urchin, herring) the mitochondria occur in a tight cluster at the base of the head, enveloping only 1–2% of the total length of the axial fibre bundle. But in more 'advanced' sperm the mitochondria spread down the axial fibre bundle to varying extents. In cock sperm a cluster of mitochondria envelops about 5% of the fibre bundle; in cockroach sperm two straight mitochondrial strands extend beside the fibre bundle almost to its tip; in some slug sperm there is a triple mitochondrial helix enveloping nearly the entire length of the fibre bundle, and in many kinds of mammalian sperm a double mitochondrial helix enveloping 20–25% of the fibre bundle. Yet in all these cases except mammalian sperm the 9+2 fibre bundle occurs virtually unchanged, and in mammalian sperm it is altered only to the extent of addition of a second ring of 9 fibres outside the first. The following account of the range of fibre patterns found in sperm tails is based mainly on work in this laboratory, supplemented where appropriate by reference to earlier investigations. It contains a description of a typical 9+2 sperm tail (sea urchin), a summary of other examples, and finally a brief account of the two known exceptions (ostracod and mammalian sperm).

Sea-urchin sperm. The tail of a sea-urchin sperm consists of a bundle of 11 fibres (Fig. 15) wrapped in a membrane which at present resolutions appears amorphous. Both *Echinus* and *Psammechinus* have been studied and no significant differences found as regards tail structure. As usual, 9 fibres (each about 250 Å. diameter) are arranged in a cylinder around 2 thinner, central fibres (about 130 Å. diameter). The outer diameter of the tail throughout most of its length is only about 0·15μ, as measured in whole sperm and in cross-sections. The terminal 5–7μ is much thinner (about 0·04μ) and superficially resembles the 'whip-lash point' of certain cilia. At its proximal end the fibre bundle passes through the middle of the disk-shaped mid-piece and ends in a centriole at the base of the head. The central pair of fibres are rather more easily disrupted in sea water or distilled water than the outer 9, but this difference in sensitivity is not so

marked as in some other sperm tails (e.g. slug and cock). The cross-striations of irregular period (500–700 Å., Fig. 16) similar to those seen by Harvey & Anderson (1943) are probably due to natural or artificial aggregation of the matrix in which the fibres are embedded. These aggregations are probably similar to those observed by Manton and her co-workers in frayed plant cilia; and are very different from the tough tail helix found in mammalian sperm, since they disappear at points where the tail frays (accidentally and without violent treatment).

*Other 9+2 invertebrate sperm tails.** From electron micrographs taken in this laboratory, Mrs A. Cameron has clearly demonstrated the existence of a 9+2 fibre pattern in several invertebrate sperm, particularly those of the cockroach and several species of slug. The cockroach sperm tail (*Periplaneta americana*) is of great interest for it falls apart into three parallel strands. The two outer strands are mitochondrial in nature; the centre strand is the 9+2 fibre bundle with 9 fibres about 500 Å. diameter and 2 of about 250 Å. diameter. Among slug sperm the axial fibre bundle (forming the core of the tail inside the helical mitochondrial strands) very clearly consists of 9+2 fibres in two species (*Arion hortensis* and *Deroceras reticulatum*; frayed tails and sections); but in a third (*Milax sowerbii*), where sections have not yet been cut, the tail usually falls apart into 18–20 subfibrils. It is reasonable to assume at present that these result from a 9+2 pattern in which, exactly as found by Fawcett & Porter for cilia, each of the outer 9 contains two subfibrils. This interpretation probably holds also for the fascinating sperm of *Ptinus tectus* (one of the spider beetles) studied by Dlugosz & Harrold (1952). Here the head is long and thin and extends far down into the 'tail' where there are also two stout strands (0·15μ thick) probably of mitochondrial origin, like those in cockroach sperm. Finally, there are 18–20 thin fibres of about 250 Å. diameter, which presumably come from a 9+2 pattern where each of the 9 is a doublet. It is appropriate also to refer here to the early work of Schmitt, Hall & Jakus (1943) on squid sperm and of Bretschneider (1950) on various invertebrate sperm.

Ostracod sperm. In collaboration with Lord Rothschild we have made preliminary studies on the remarkable sperm of fresh-water ostracods, particularly in the genus *Notodromas*. The sperm are about 1 mm. long, the head being 0·45 mm. long and 0·25μ wide and the tail 0·55 mm. long and 1·8μ wide (though it is impossible to specify lengths exactly since head merges into tail). The tail consists of a six-strand flex surrounded by a thick sheath which bears the imprint of the coiled strands inside it (Fig. 17). Four of the strands are about 0·65μ thick and are presumed to be non-contractile mitochondrial derivatives. Much denser than these broad

* See addendum.

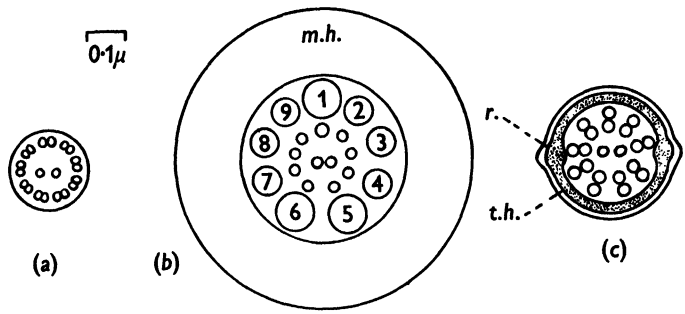
strands are two narrow strands (0·18μ thick) which themselves appear to contain helical structure and need further study. These narrow, dense strands are provisionally interpreted as the contractile elements. We have not yet sectioned the sperm, but so far as is known at present it contains no straight fibres comparable with the 9+2 fibres elsewhere.

Sperm of vertebrates other than mammals. Jakus & Hall (1946) showed the 9+2 pattern in *Paramoecium* cilia, but did not then realize its significance; the first clear demonstration of the 9+2 pattern in flagella was by Grigg & Hodge (1949) in the cock. The 9 thicker fibres are about 450 Å. diameter. The two thinner fibres are about 300 Å. thick and much more easily disrupted by distilled water and the like. Recently we have found the same sort of arrangement in frog sperm; sections of mid-piece and tail are shown in Fig. 18. Lowman (1953) states that in salmon sperm, not only are there 9+2 fibres, but also that each fibre itself consists of subfibrils —5 in some fibres and less in the others. Between 9 and 11 fibres have been found in various fish sperm by Rotheli, Roth & Medem (1950) and Hug, Lippert & Fischer (1953). Reptilian sperm have not yet been thoroughly investigated.

Mammalian sperm. The outstanding feature of mammalian sperm is the addition of an extra ring of 9 fibres not all of equal thickness (Bradfield (1953) and Bradfield (1955), where the studies of Bretschneider (1949) and Randall & Friedlander (1950) are also discussed). In bull, boar and ram sperm mid-pieces, for example, there are two centre fibres, each of about 180 Å. diameter, around them a ring of 9 fibres of similar diameter, and around them a ring of 9 much thicker fibres, three of which are about 1000 Å. in diameter and the other six about 700 Å. in diameter. The disposition of thicker and thinner fibres is indicated in Text-fig. 3*b* and Fig. 19. The thickest fibres, in particular, often appear wedge-shaped on account of their close packing. Inside the outer fibres it is often possible to detect signs of numerous subfibrils; these have not yet been measured accurately, but are of the order of 75 Å. in diameter. An important point is that the thickest fibres are in positions 1, 5 and 6, so that the axis of symmetry can only be the diameter at right angles to the central pair, bisecting fibre 1 and passing midway between 5 and 6; it cannot be the diameter passing through the central pair. (Very occasionally, fibre 2 or 9 appears to be as thick as fibre 1, so that there are 4 thicker fibres, in which case the diameter containing the central pair is a possible axis of symmetry (Text-fig. 4*b*); this is rare, having been observed in only 5% of mid-piece transverse sections, whereas the greater thickness of fibres 1, 5 and 6 has been observed in 100% of sections.) In the tails of mammalian sperm the 9 thick outer fibres become much thinner and closely applied to their inner partners (Text-fig. 3*c* and

Fig. 20). This arrangement of 9 doublets is reminiscent of that found by Fawcett & Porter in cilia, except that there the twin strands in each of the 9 ciliary doublets lie side by side (tangentially) on the same circumference (Text-fig. 3*a*) instead of one inside the other on the same radius (radially).

The fibre pattern of the mammalian sperm tail is, in effect, an elaboration on the 9+2 theme and can conveniently be represented by the formula 9+9+2. Its evolutionary origin is quite unknown. It might be that the tangential doublets of an ancestral sperm tail rotated through 90° so as to become radial and then the outer member of each hypertrophied in the mid-piece where it was surrounded by the energy-producing mitochondrial



Text-fig. 3. Diagrammatic transverse sections of cilia and flagella showing arrangement of fibres. (*a*) Typical 9+2 array found in all cilia which have been investigated and in all flagella investigated, except mammalian sperm tails and ostracod sperm tails (if the latter are called flagella). In all the cilia and several of the flagella each of the outer 9 fibres consists of 2 subfibrils as shown. (*b*) Mid-piece of mammalian sperm tail showing 9+9+2 array. Among the 9 thick outer fibres, 3 (numbers 1, 5 and 6) are thicker than the rest so that there is only one possible axis of symmetry, namely, the north-south diameter (*m.h.* = mitochondrial helix). (*c*) Tail of mammalian sperm. Each of the 9 thick fibres of the mid-piece has become thinner and closely applied to its partner in the inner ring, producing 9 radial doublets, in contrast to the tangential doublets in (*a*). *r.* = rib on tail helix (*t.h.*).

helix. To check this possibility it is desirable to discover (by study at higher resolutions) whether or not each of the inner 9 fibres is a tangential doublet, like the 9 doublets in cilia, as well as to investigate marsupials and monotremes. The mystery of the origin of the mammalian sperm's 9+9+2 pattern is deepened by the fact that both in developing sperm (Challice, 1953) and in the distal part of the mature sperm mid-piece there is evidence that each of the three thickest fibres in the outer ring arises from lateral fusion of a tangential pair of fibres, i.e. that the ring of 9 is produced by appropriate fusions in a ring of 12.

In all mammalian sperm so far studied in this laboratory (bull, ram, boar, human, opossum, dog, rabbit) the axial bundle of 9+9+2 fibres is surrounded in the tail by a helix, containing structural protein, down which run two ribs, or fins (Fig. 20), which are on the same diameter as the central pair. This helix has rather curious solubility properties. It is little

affected by normal acetic or hydrochloric acids, but is completely dissolved by N/20 sodium hydroxide (in about ½ hr. at 37° C. or 1 hr. at 20° C.). The axial fibres are dissolved almost as fast as the helix, but by frequent sampling during treatment sperm can sometimes be found with the helix removed but much of the axial fibres remaining (Fig. 21). Stronger alkali (N/10 and N/2 have been investigated) is correspondingly more effective. M/2 sodium thioglycollate produces a very marked inhibition of the alkali effect. A less marked protective effect is produced by M/2 sodium chloride, so that some of the protective effect of thioglycollate is presumably due simply to salt concentration. The rest may be due simply to buffering effect, but at any rate it is evident that the helix is composed of an extremely resistant material, for after 3 hr. in a solution prepared by taking M sodium thioglycollate (pH 7) and adding an equal quantity of N-NaOH the helix is somewhat swollen but not dissolved, in spite of the fact that it is initially so finely divided. Whether, like true keratins, it eventually dissolves after many hours in this mixture (similar to that recommended by Goddard & Michaelis, 1934) is now under investigation. Full study of the effects of any one treatment is a large task, since many sperm on many grids must be examined in order to obtain a truly representative picture. The axial fibres dissolve in all these cases despite the presence of M/2 sodium chloride or thioglycollate, leaving an empty or partially empty helix depending on the duration of treatment. Exactly the same result is obtained with 6 M urea, which leaches out the axial fibres, but has much less effect on the helix. The way in which the helix withstands such reagents argues against its being a contractile element and in favour of its being a skeletal binding element composed of some very resistant protein. (The whole tail is dissolved by trypsin and pepsin.) Certainly mammalian sperm tails are extremely resistant to disintegration—much more so than the tails of many other sperm. No other fibre-binding structures, beside the mitochondrial and tail helices, have been unequivocally demonstrated in mammalian sperm, but there certainly must be some binding structure uniting the inner and outer members of each of the 9 radial doublets in the tail. In addition, there is in all flagella, as in cilia, an interfibrillar matrix to which little attention has so far been devoted.

THE BASIC MACHINERY OF CILIA AND FLAGELLA AND THE SIGNIFICANCE OF ASSOCIATED STRUCTURES

One of the most striking features emerging from the foregoing brief survey of our present knowledge of ciliary and flagellar structure is the contrast between the great constancy of the fibre patterns in cilia and flagella (with

a few exceptions) and the great variation in the nature and arrangement of the associated structures. In any attempt to discover whether or not there is a minimum basic machinery essential for ciliary and flagellar activity and common to all true cilia and flagella, both cytological and physiological evidence must be taken into account. From the latter point of view there are various studies on how the beating of cilia and flagella is affected by cuts at various levels in the cells to which they are attached (Gray, 1928; Worley, 1941). The general conclusion from such work is (1) that the minimum structures necessary for the beating of an individual cilium or flagellum are the basal granule (or corpuscle) and, of course, the vibratile process itself (consisting of fibre-bundle embedded in matrix and surrounded by membrane); and (2) that other associated structures such as rootlets, other fibres in the apical cytoplasm round the bases of cilia, and mitochondria are non-essential from this point of view. Such a conclusion is entirely in accordance with the cytological evidence in that basal corpuscle plus vibratile process are the only features common to all cilia and flagella. The status of the various non-essential accessory structures associated with basal corpuscles is briefly considered in the remainder of this section.

Rootlet fibres are widespread among cilia, but Fawcett & Porter find no sign of them in the ciliated epithelium of mouse and human oviduct, and most sperm lack anything comparable with them. They cannot be essential for movement. Rootlets may be important for co-ordination as shown by the micro-dissection experiments of Worley (1941) on *Anodon* and as suggested by the cross-over rootlet patterns of *Mytilus* laterofrontal cilia. The fact that rootlets can seldom be traced farther than the nucleus raises the possibility that they may terminate on or near the centrosome (from which basal granules are known in some cases to be derived). Such connexions certainly exist in some Protozoa. Rootlets may also provide anchorage—particularly in those cases (e.g. *Pleurobrachia*) where they end in many fine branches comparable, on a micro-scale, with the roots of higher plants. The cross-striations in rootlets are of unknown significance, but are of course reminiscent of those in collagen and fibrin.

The abundant *cytoplasmic fibres* observed by Fawcett & Porter just beneath the lumen surface of some ciliated cells presumably have a stiffening function. They are poorly developed in many ciliated epithelia containing good rootlets, but there is not a clear inverse relationship between rootlets and these superficial fibres because both are well developed in, for example, the ciliated cells of frog pharynx and oviduct. Both because it is absent in some cases and because, when present, it does not make obvious connexions with the cilium, this fibrous component is unlikely to be an important part of the vibratile mechanism.

Mitochondria are commonly associated with basal corpuscles; and, indeed, during the evolution of animal sperm mitochondria have in many cases come to envelop a considerable length of the fibre-bundle in the actual flagellum itself—either as a cluster of globular elements or as helical strands. The oxidative enzyme content and non-fibrous nature of mitochondria lead one to suppose that they are solely concerned with providing the energy for beating and are not directly capable of causing movement. In many ciliated cells, the mitochondria, though plentiful, are not even very closely associated with the basal corpuscles.

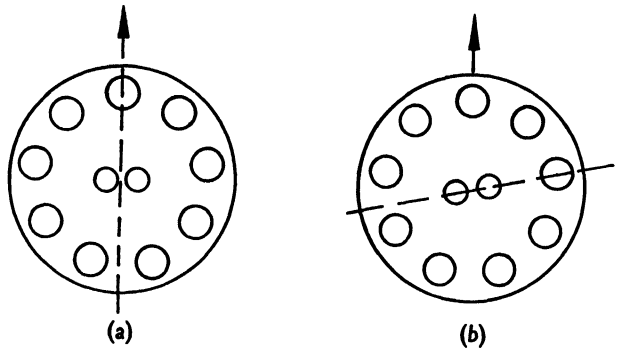
Since *helical strands* have not been found in cilia they cannot be part of any basic vibratile mechanism common to flagella and cilia. Moreover, there is good reason for thinking that even in those flagella where helical strands are found they are not contractile. In many cases (e.g. in the sperm of certain gastropods and birds and in the mammalian sperm mid-piece) they are mitochondrial threads concerned in energy production. The tail helix in mammalian sperm appears to consist of structural protein and serves as a skeletal element preventing the fibre-bundle from falling apart so easily as it does in many sperm tails which lack a helix. There is, in fact, no clear evidence at present for the existence of helical binding strands in any other animal flagella; the interesting work of Brown (1945), for example, cannot be taken as proving the existence of a helical strand round the flagellum of *Euglena*.

Only the basal corpuscle and the 9+2 bundle of straight fibres with its matrix and membrane are, therefore, constant and essential parts of the vibratile apparatus in all flagella and cilia. This does not imply that isolated fragments of the fibre-bundle, separated from the basal corpuscle, are incapable of contracting, but simply that the basal granule is an essential part of the machinery for producing the normal integrated beat of the fibre-bundle in the single living flagellum or cilium.

POSSIBLE MECHANISMS OF FLAGELLAR AND CILIARY MOVEMENT

If the basal corpuscle together with the 9+2 fibre-bundle and its matrix and membrane constitute the basic machinery for vibratile movement, the question to be considered next is how they produce the observed movements. The conclusion reached in the last section that rootlets and fibrous components in the surrounding cytoplasm are not essential parts of the machinery argues against any passive wagging of the cilium or flagellum from its base by means of contractile structures acting on the basal corpuscle. Further arguments against such a possibility are provided by the

following considerations: (1) The orientation of the basal corpuscle *and basal micron of the cilium* as seen in thin sections does not vary appreciably, or in any constant manner, during different phases of contraction in the lateral and laterofrontal cells of *Mytilus* (Figs. 7, 11 and 12). (2) As Gray has pointed out in detail, it is extremely probable that contractile energy is added along much of the length of the flagellum or cilium. Two arguments here are: (*a*) in some cilia the angular velocity of the tip is greater than that of the base, so that the whole cilium becomes concave forwards during the effective stroke; (*b*) not all waves passing down a flagellum undergo a reduction in amplitude and 'a moving wave cannot provide the energy for



Text-fig. 4. The two possible axes of symmetry (broken lines) of the 9+2 configuration. The line joining the two central fibres may be either at right angles to the axis of symmetry (*a*), or coincident with it (*b*). The axis itself can bisect any of the outer 9 so long as they are all identical, but in mammalian sperm it has only one possible position—bisecting the single thicker fibre in the outer ring (number 1 in Text-fig. 3*b*). In cilia and non-mammalian sperm tails it is impossible to distinguish with certainty between (*a*) and (*b*), but in general (*a*) seems to fit best and this would also be homologous with the arrangement in mammalian sperm tails. The direction of beat in those cilia where it has been identified in sections is indicated by the solid arrow.

propelling an organism and at the same time pass on with unreduced amplitude, unless the energy being lost is continually being replaced as the waves move along' (Gray, 1928). From several considerations therefore it is reasonable to conclude that the moving part (fibre-bundle with its matrix and membrane) itself produces some, if not all, the force responsible for the movement. Before going on to discuss how it does so, it is necessary to consider the precise arrangement of the 9+2 fibres as seen in transverse and longitudinal section.

A circle containing a ring of 9 equidistant smaller circles with 2 circles in the middle (Text-fig. 4) can have either of two possible axes of symmetry. The latter must bisect one of the outer 9 circles. The centres of the 2 middle circles can then lie either equidistant from the axis and on a line at right angles to it as in Text-fig. 4*a* or on the axis as in Text-fig. 4*b*. If we then

imagine the diagram to be a cross-section of a cilium or flagellum and add the fact that the direction of beat (in some cilia at least) is approximately at right angles to the line joining the central pair, with an odd fibre on the side of the effective stroke, we find that there is one obvious beat direction in Text-fig. 4*a* coinciding with the axis of symmetry; and in Text-fig. 4*b* there is one obvious beat direction at 80° to the axis of symmetry through the 2 middle circles. Finally, it is possible that the two centre fibres are so arranged that there is no axis of symmetry. The angle between the line joining the centre pair in Text-fig. 4*a* and the line joining the centre pair in Text-fig. 4*b* is only 10°, and it is impossible at present to distinguish with certainty between the various possibilities in view of the minor distortions which can occur during preparation and sectioning. In the following discussion it will be assumed that cross-sections of cilia and flagella conform to Text-fig. 4*a*, which is best in accordance with micrographs of cilia taken here and elsewhere and is the only symmetrical pattern possible for mammalian sperm, where Text-fig. 4*b*, for example, is impossible. However, the very small difference of alignment in the centre pair between Text-fig. 4*a* and *b* is actually unimportant below, since it is assumed that only the outer 9 are contractile.

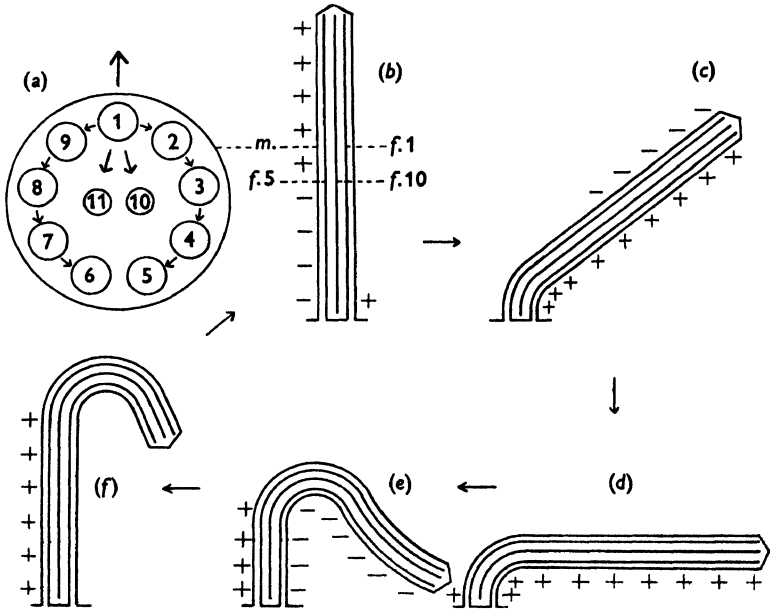
The question whether the 9+2 fibres run straight up the cilium or flagellum, or whether any of them spirals, as do the supposedly contractile strands in spirochaetes and ostracod sperm, is also hard to settle. Longitudinal sections, both of cilia and of sperm tails, show clearly that the fibrils do not spiral several times and suggest that they run straight. Yet it is difficult to exclude the possibility that they run in a very open spiral passing, say, only once round the cilium from base to tip, because we seldom obtain longitudinal sections extending along the cilium for more than a few micra. It seems most unlikely that the centre pair spiral in cilia because of their constant relation to the direction of beat in the examples studied; and in mammalian sperm it is clear that if any fibres spiral all 9+9+2 must do so and with the same wave-length. In general the evidence in true flagella and cilia is everywhere in favour of straight fibres.

If the fibres spiral, then both two-dimensional and three-dimensional waves can be generated, in a flagellum for example, simply by pulling on the fibres at their bases—as the elegant models of G. I. Taylor have shown. But if the fibres run straight, as seems to be the case, then it appears that the observed phenomena are most likely to be explained in terms of localized contractions propagated along the fibres. The hypotheses advanced below will be based on the following *assumptions*: (1) The 9 outer fibres are capable of propagating localized contractions. (2) The 2 centre fibres are not contractile, but are specialized for more rapid conduction (in cilia—

their function is enigmatical in flagella). (3) The stiffness of a cilium or flagellum (like that of an earthworm or a sausage-shaped balloon) depends on the fluid pressure inside it—as envisaged by Gray (1928, 1930), and as suggested by the wrinkled membrane which almost always appears in fixed and dehydrated material whether the fixative is hypo- or hypertonic. (4) The impulses producing contraction arise rhythmically at a point in the basal corpuscle (or ring, or plate) directly beneath one of the 9 outer fibres and each impulse travels round to the other 8 fibres and then dies out. (5) Contraction (or relaxation) of each fibre involves interaction between its contractile protein and some energy-rich small molecule; the latter is destroyed in the process and regenerated by the mitochondria, which are commonly clustered round the bases of cilia and flagella and may even be wrapped round an appreciable length of the fibre bundle in more complex sperm tails.

Cilium. The kind of ciliary movement revealed by Gray (1930, pl. 20; 1931, fig. 184) in the abfrontal cilia of *Mytilus*—with the cilium more or less vertical at the beginning of the stiff effective phase and more or less horizontal at the beginning of the limp recovery phase—might be produced by the following sequence of events. At the beginning of the effective stroke (Text-fig. 5*b*) an impulse arises in the basal corpuscle under fibre 1, fires off a propagated contraction up that fibre and spreads with equal speed in both directions round the ring firing off fibres 2 and 9. At the same time the impulse is picked up by the central pair of fibres (which are specialized for conduction, but are not connected to the basal plate). It is rapidly conveyed by them throughout the entire length of the cilium, thus stimulating the upper regions of fibres 1, 2, 9, 3 and 8 to contract. This stiffens the 'leading edge' of the cilium and prevents it from being bent back (so as to be concave backwards) by the resistance of the medium during the effective stroke (Text-fig. 5*c*). Indeed, there is a possibility of the reverse—of the cilium being bent concave forwards—so that the tip moves with greater angular velocity than the base, as has sometimes been observed. The impulse conducted by 10 and 11 does not stimulate 4, 7, 5 and 6 because they are in process of relaxation, and hence refractory, following their contraction during the recovery phase (see below and Text-fig. 5*f*). The impulse circulating round the basal corpuscle has meanwhile spread past 2 and 9 to 3 and 8 and the combined contractions of 1, 2, 9, 3 and 8 (particularly of their basal regions, see p. 327) bend the stiffened cilium into the horizontal position and complete the effective stroke (Text-fig. 5*d*). The continued spread of the impulse round the basal corpuscle next fires off fibres 4 and 7 and then 5 and 6, whose relaxation is now complete. Contractions spread up these fibres at their natural speed of propagation and

ll the cilium up into the vertical position (recovery phase) through a series curves only two of which are shown in Text-figs. 5*e* and *f*. The pulses at the bases of fibres 4, 7, 5 and 6 are not picked up and spread to upper parts of the cilium by the more rapidly conducting central pair and 11) because the latter are in a refractory state following their con-



xt-fig. 5. Hypothetical mechanism for ciliary movement. (*a*) T.S. cilium. The impulse ising contraction of the fibres is assumed to originate under fibre 1 and spread both ind the ring of 9 fibres and to the central pair, as indicated by arrows inside the cilium. e external arrow indicates the direction of beat. (*b*) Side view of cilium beating in plane paper showing membrane (*m*), fibre 1 (*f*. 1), fibre 5 (*f*. 5) and fibre 10 (*f*. 10). The cilium it the beginning of the effective stroke. An impulse arising under fibre 1 has just pro-ed a propagated contraction (indicated by plus sign) at the base of this fibre. (*c*) The pulse is spreading round the basal apparatus (not indicated) and has also spread to the tral fibres which are specialized for conduction and rapidly convey it up the cilium ising fibres 1, 2, 9, 3, 8 to contract (plus signs) almost simultaneously throughout their gth (only fibre 1 shown). As a result the cilium is bent forward as a stiff rod and is not ormed by the resistance of the medium so as to be convex forwards. (*d*) The end of the ctive stroke. The impulse spreading round the basal ring is reaching fibres 4, 7, 5 and 6 l initiating contraction at their base (plus sign). (*e*) The contractions in 4, 7, 5 and 6 ead slowly up these fibres at their natural rate of propagation and are not spread by the idly conducting central fibres because the latter are now in their refractory state. res 1, 2, 9, 3, 8 are relaxing (minus sign) and hence also refractory. Owing to the con-tion being propagated more slowly than in the effective stroke, the cilium returns to the tical through a series of curves and not as a stiff rod. (*f*) Contraction continuing spread up fibres 4, 7, 5 and 6; completion of recovery stroke. Relaxation complete (no n) in fibres 1, 2, 9, 3, 8 which are therefore ready to commence the effective stroke in (*b*). (*b*) and (*c*) fibres 4, 7, 5 and 6 which have brought about the recovery stroke are relaxing cept perhaps in their distal regions) and are hence refractory and not affected by the pulse conducted by the central fibres. The cilium is arbitrarily shown as vertical at the l of the recovery stroke and beginning of the effective stroke. In fact, however, it is riously likely on the mechanism postulated here that the cilium may be pulled over to left of vertical during recovery (as occurred in the cilia photographed by Gray, 1930).

duction in the first part of the cycle. This is the reason for the difference in form between the stiff effective stroke and the curving recovery stroke. The impulse in the basal ring dies out between fibres 5 and 6 and a new one arises under fibre 1 which starts another cycle.

There are so many unknowns that it is almost impossible to make useful calculations regarding the *tensions* needed in individual fibres for the production of the observed movements according to the above hypothesis. Simple geometry, however, shows that the degree of *shortening* required is small. If, as in Fig. 12*b*, the bending through 90° occurs mainly in a 5μ length of the cilium (near its base) and the radius of the cilium in cross-section is 0·1μ, then the maximum shortening (i.e. in fibre 1 of Text-fig. 5*a*, the odd fibre on the side toward the effective stroke) is only π%. In fibres 2, 9, 3 and 8 of Text-fig. 5*a* shortening would be less.

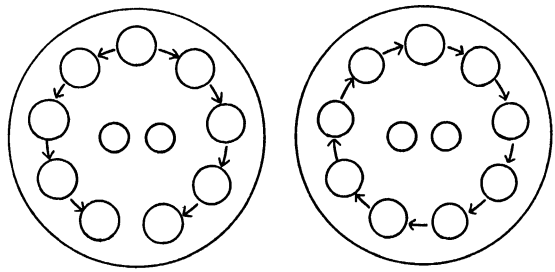
The concept of impulses arising rhythmically at a point in the basal corpuscle and spreading round it raises the question whether any structural polarity (suggesting a possible point of origin for an impulse) can be detected in basal corpuscles. In several cases Fawcett & Porter have clearly demonstrated such a polarity in the presence of a lateral knob or foot on the basal corpuscle; and in their fig. 25, showing frog pharyngeal cilia, it appears that the knob and foot are in the plane at right angles to the central pair, i.e. are in the plane of beating, as required of the site initiating the impulse in the hypothesis of ciliary movement discussed above. On the other hand, polarity has not yet been detected in basal corpuscles of *Pleurobrachia* and *Mytilus*, though in the latter the ciliary rootlets are in some cases attached very much on one side of the basal corpuscle.

Flagellum. (*a*) Two-dimensional waves might be produced if the impulse spreads both ways round the ring from fibre 1 (dying out between fibres 5 and 6, or continuing round, or being reflected back), and is *not* picked up and spread rapidly along the flagellum in the manner postulated above for cilia. Such a mechanism with nine equal fibres (Text-fig. 6*a*) seems unsatisfactory in that tension developed in fibres 3 and 8 (and to a less extent in 2, 9, 4 and 7) is somewhat ineffective in producing bending of the flagellum. In this respect, therefore, it is interesting to recall that in the outer ring of 9 fibres in the mammalian sperm mid-piece, fibres 1, 5 and 6—the most effectively placed fibres according to the above scheme—are much thicker than the 6 less effectively placed fibres (3, 8, 2, 9, 4 and 7), as if the latter were becoming vestigial in the course of evolution (Text-fig. 3*a*). An obvious weakness in the two schemes for flagellar movement is the fact that neither suggests any specific function for the central pair of fibres.

(*b*) Three-dimensional, corkscrew-like waves might be produced by an impulse arising in the basal corpuscle beneath fibre 1, and then spreading

round the corpuscle in one direction only so as to fire off propagated contractions along each fibre in turn (Text-fig. 6*b*). The distance between the localized contractions in successive fibres (and hence the form of the waves generated) would depend on the time taken for the impulse to travel round the ring—assuming all nine fibres to have identical properties.

Space does not permit detailed discussion of the above hypothesis of ciliary movement in the light of the extensive experimental work which has been carried out on this subject (Gray, 1928; Heilbrunn, 1943; Prosser, 1950). In general there are no instances where the hypothesis is clearly incompatible with experimental results (though its chemical implications cannot be checked since no specific suggestions have been made above regarding the chemical mechanisms of contraction and conduction). In the



Text-fig. 6. Hypothetical spread of impulse during flagellar movement. (*a*) As in the cilium the impulse arises under fibre 1 and spreads each way round the fibre ring. It is not conducted, however, by the central pair. Hence localized contractions spread up the fibres at their natural speed of propagation; this would generate two-dimensional waves whose form would depend on the rate of spread of the impulse round the ring. (*b*) Three-dimensional, corkscrew-like waves would be produced if, as shown here, the impulse were to spread only one way round the basal ring (and not be conducted by the central pair).

mechanical studies of Carter (1924) it was observed that a 'cilium' was stiff when pushed through the effective stroke with a needle after natural beating had ceased. This appears superficially to contradict theory which implies that when contraction in the 9 fibres has ceased the cilium should be limp and flexible in more or less any position. Carter points out, however, that the observed behaviour is a property only of complex bunches of 4–5 cilia and that individual cilia are limp when pushed in either direction as would be expected from theory. Authentic cases of ciliary reversal might be explained in terms of the impulse arising under fibres 5 or 6, or midway between them (instead of under fibre 1—Text-fig. 5*a*), spreading to the central pair as usual, and then circulating each way round the basal ring in the direction opposite from that in which it normally passes. Pendular movement (with the cilium stiff in both strokes) might be due to a shorter refractory period in the central pair, with the result that they

conducted again by the time the impulse circulating round the basal ring had reached fibres 4, 7, 5 and 6, so that these fibres, like those producing the effective stroke, would be thrown into contraction throughout their length and produce a stiff stroke. Clearly the time relations of conduction round the basal corpuscle and up the two kinds of axial fibre would be very important, but unfortunately no information is available here. The exact functional significance of the central pair poses a very interesting and difficult problem. In the preceding discussion they have been assumed to be specialized for conduction, as suggested by Grigg & Hodge (1949), and to act (in cilia) rather like the giant nerve fibres of an earthworm, in causing contraction almost simultaneously along the entire length of fibres 1, 2, 9, 3 and 8 in Text-fig. 5*a* so that the cilium is stiff in the effective stroke and not bent back by the medium. They can hardly have an important contractile function since, apart from their ineffective mechanical position in the centre of the cilium, they are unattached at both ends. And it seems unlikely that they have an important skeletal function since (in flagella at least) they tend to be more easily dissolved and lost than the outer 9. The view that some part of the cilium is specialized for conduction is in accordance with the incorporation of cilia into so many kinds of sense organs in both invertebrates and vertebrates.

As regards the fine structure and possible mode of shortening of the 9 peripheral fibres, little definite can be said. As indicated in the sections concerned with ciliary and flagellar structure, there is evidence that each of the 9 consists of subfibrils: 2 per fibre in cilia and many flagella; many more than 2 per fibre in the 9 thick outer fibres of mammalian sperm mid-pieces; and possibly 4–5 per fibre in salmon sperm. Hence there is ample scope for contraction by one subfibril sliding up on the other, without either shortening, in the manner suggested for muscle by Hanson & Huxley (1955). In this connexion it may be significant that in the sperm tail of the spider beetle (according to the interpretation given earlier) one strand in each of the 9 doublets is shorter than the other strand in the same doublet by about 1·5μ or 3% of its length (Dlugosz & Harrold, 1952, fig. 4). There is also the conventional possibility of contraction by shortening of individual subfibrils, produced either by folding linear polypeptide chains or tightening coils in a helical polypeptide chain. Judging from the micrographs and tracings of Fawcett & Porter the two subfibrils themselves run straight along each fibre and not in a helix. It is certainly the case in the mammalian sperm tail that the individual fibres in the *radial* doublets run straight and do not wind round each other—otherwise of course they would not always appear as radial doublets. Manton & Clarke (1952) have shown clearly in cilia of the moss, *Sphagnum*, that the two subfibrils in each of the 9 doublet

fibres are embedded in some sort of matrix which normally hides their presence and makes the fibre appear as a single homogeneous thread. Presumably the same is true of doublet fibres in animal cilia and flagella, and variations in the nature and abundance of the matrix material could explain variations in the ease with which the 9 fibres fall apart into 18 subfibrils—the difference, for example, between sperm of the slug *Milax sowerbii*, where the tail falls apart directly into the 18 subfibrils, and the very similar sperm of the slug *Arion hortensis* where only the usual 9 peripheral fibres appear in a frayed tail and their doublet nature is not at once apparent (Cameron, 1954).

Quite apart from such variations in the amount of matrix material (which will affect the contours of the fibres) there is, in mammalian sperm for example, a distinct taper in each of the outer 9 thick fibres—a sharp taper at the junction of mid-piece and tail and a more gradual taper beyond this point, in the tail. Presumably therefore the tension which can be developed per micron of fibre is appreciably greater in that part of the fibre which lies in the mid-piece than in that part which lies in the tail. Whether such tapering occurs in the fibres of other flagella and of cilia is uncertain; but even if it does not, the tension developed per micron of fibre at the base is likely to be stronger than in the distal regions, because there will be at the base a higher concentration of the energy-rich substance, which, according to the scheme put forward above, is manufactured by the mitochondria and diffuses into the cilium or flagellum for use during contraction.

THE ORIGIN AND CONSTANCY OF THE 9+2 PATTERN AND THE SIGNIFICANCE OF DEPARTURES FROM IT

The constancy of the 9+2 pattern of fibres stands in striking contrast to the wide variation in the movements of flagella and cilia and in the form and arrangement of their accessory structures such as rootlets and mitochondria. The only hope of tracing the origin of the pattern (from a bunch of separate bacterial flagella, each equivalent to one of the 11 fibres in a 9+2 flagellum) lies in exhaustive study of the flagella of *Protista*, for the blue-green algae which might be expected to shed light on the problem are unfortunately devoid of flagella. In the larger and more complex motile bacteria, such as spirilla and spirochaetes, there are often intricate arrays of flagella (each flagellum being of the same order of thickness as one of the fibres in a 9+2 flagellum). There are bunches and bundles, which even simulate multifibrillar animal and plant flagella, in, for example, the *Spirillum* studied by Houwink (1953) and the *Salmonella* photographed by Pijper & Abraham (1954). But in no such case so far described is there a membrane enveloping

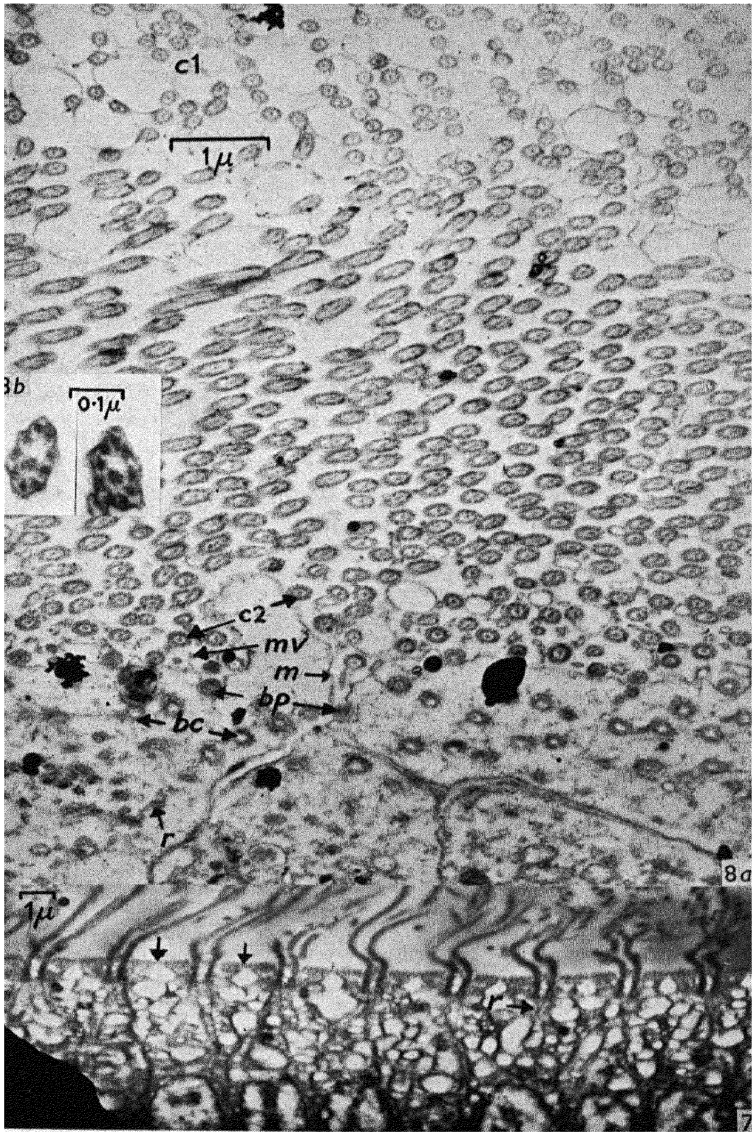
two or more of the bacterial flagella and there appears to be wide variability of fibre number in such bundles, especially among spirochaetes. These all possess the same structural plan—a twin flex where one strand is the body and the other is the bundle of flagella (Bradfield & Cater, 1952). But the number of strands in the helically wound flagellar bundle may be 1 (*Leptospira*), 4, or about 12, or about 20 (species of *Treponema*); or several hundred (*Cristispira*).

There are at least three possible ways of attempting to explain the remarkable constancy of the 9+2 pattern:

(1) It may result from the intrinsic properties of the proteins forming the 11 fibres, such that during fibrogenesis only the observed configuration can be produced by the centriole or basal corpuscle. This seems unlikely, especially in view of the known changes in number by fusion which occur during mammalian spermiogenesis. But insufficient is known of the process of fibrogenesis and of the proteins concerned to settle the matter.

(2) It may have been a chance integration of 11 bacterial flagella (or 10 if the central pair is only a loose doublet), which subsequently became a rigid character for some genetical reason and not because it constituted the only solution from the mechanical point of view. The argument here might be that the centriole which generates the 9+2 fibres is known in some cases to be derived from the centrosome, which in animals plays an important part in cell division; that genetic stability is important for the centrosome (lest lethal mitotic and meiotic aberrations be produced); and that therefore the 9+2 pattern, once attained and found satisfactory for the reasons set out in (3) below, has tended to remain unchanged.

(3) The third possibility is that the 9+2 pattern is the only possible mechanical solution. This cannot be true in its simplest, unqualified form, in view of the known departures in mammalian sperm, and in ostracod sperm (though the motility achieved by the latter is not great). If the two centre fibres were each the same thickness as the peripheral fibres and the latter touched each other, there would be good geometrical reason (Manton & Clarke, 1952) for believing that 9 has unique significance, because in that case the two central fibres would just touch the outer ring on each side. But the two centre fibres are *not* the same thickness as the outer 9 and do not touch them (unless there is gross shrinkage of about 50% in fibre width, which seems unlikely since there is not much shrinkage in the outside diameters of cilia and flagella, so far as it is possible to measure accurately). Allowing the centre fibres to be somewhat thinner than the outer fibres, it is perfectly possible to construct a cross-section possessing only 8 outer fibres. On the other hand, allowing that the centre fibres do not touch the outer fibres it is perfectly possible to fit in 10 outer fibres. 9 belongs to the



For explanation see p. 333

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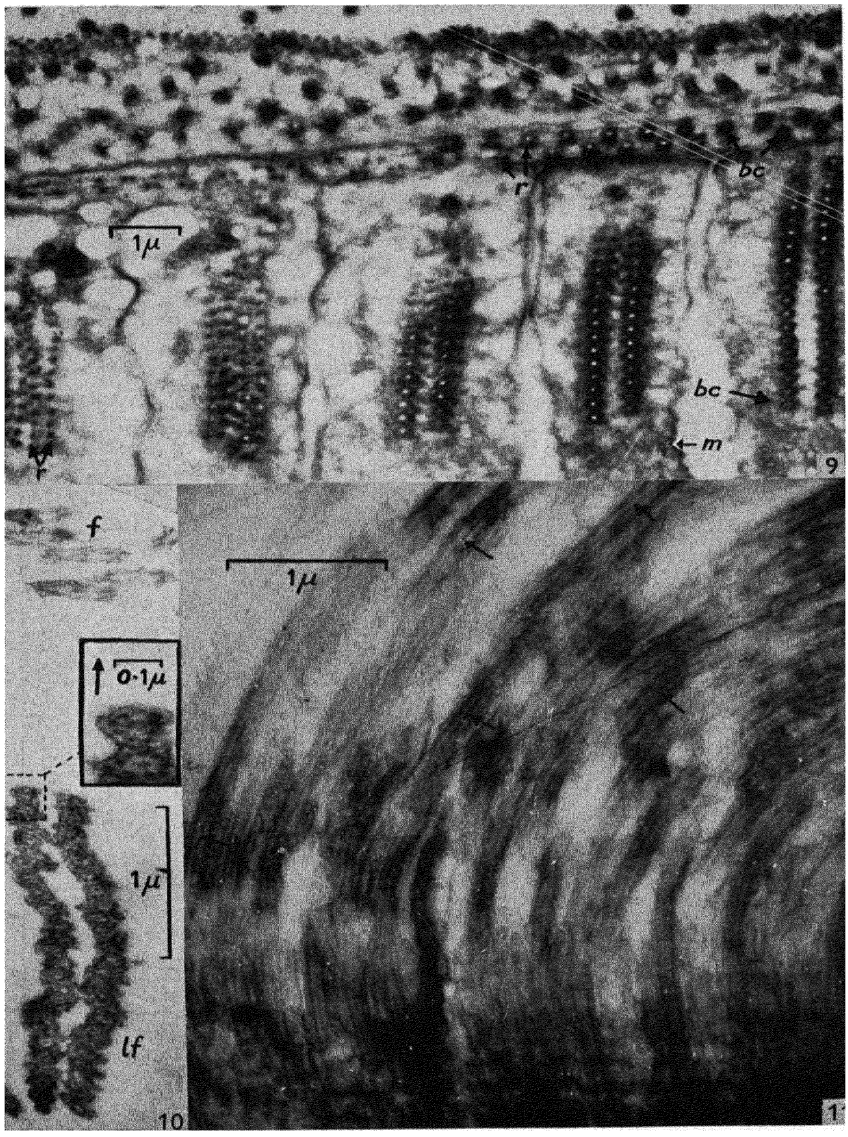

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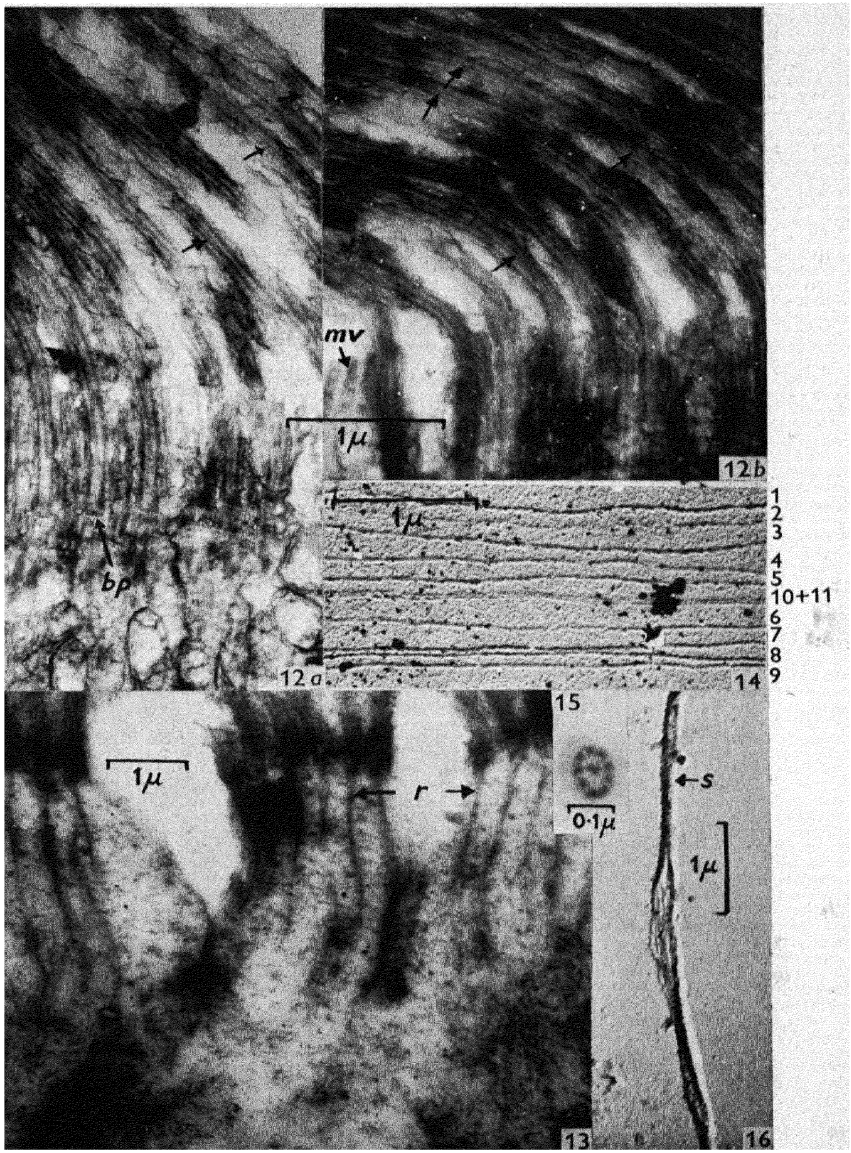

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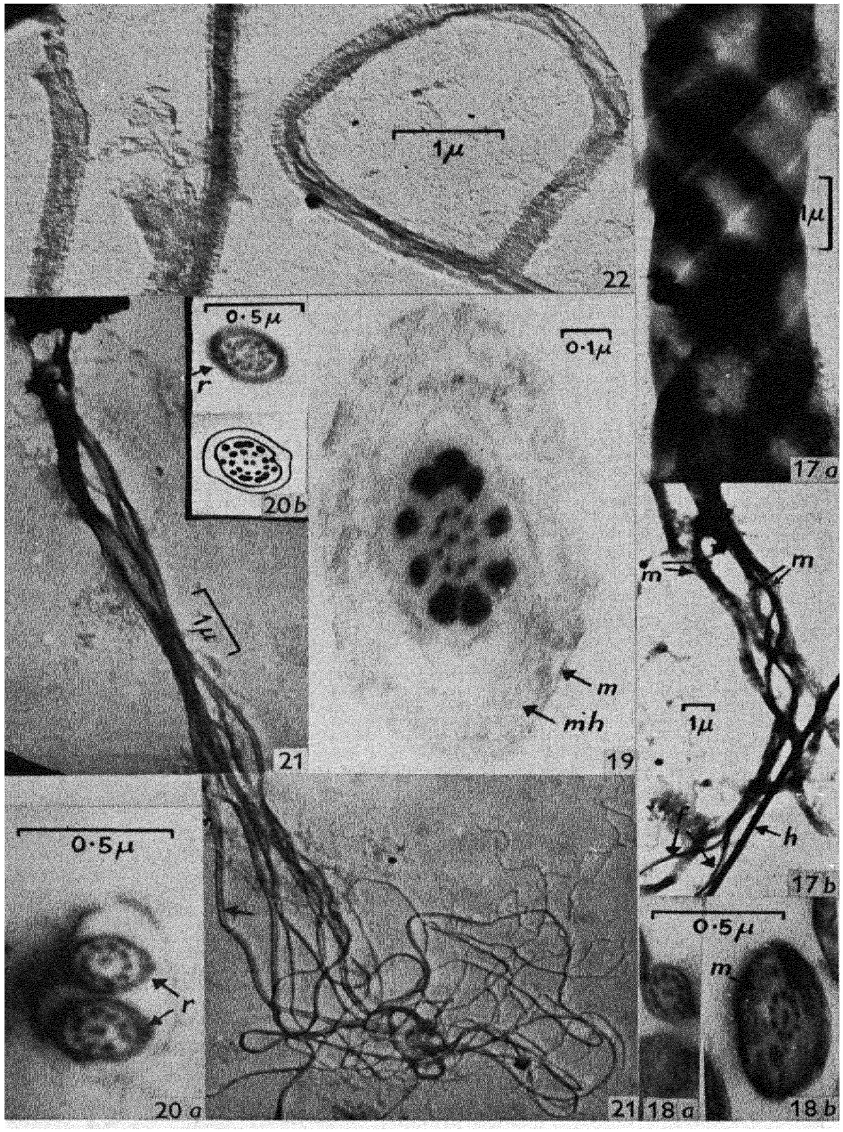

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$4n+1$ series of odd numbers (as opposed to the $4n-1$ series) whose odd 'half' is greater than their even 'half'. But it is difficult to see any significance in this because if, as has been assumed in Text-fig. 5*a*, the 9 fibres are equidistant from each other, the total bending moments of the top 5 fibres—numbers 1, 2, 9, 3, 8—about the neutral axis (the diameter through the central pair) equal the total bending moments of the bottom 4 (4, 7, 5, 6). There is no question, therefore, of the 5 fibres concerned in the effective stroke being able to exert a stronger pull than the 4 concerned in recovery (assuming all fibres to develop equal tensions). All that can be said is that 9 is of about the right magnitude from the geometrical point of view; and that unlike its even neighbours it offers a system where the impulse for contraction can arise under one fibre (number 1 in Text-fig. 5*a*) and, after spreading each way round the basal corpuscle, can end under separate fibres (5 and 6) instead of on one fibre. The significance of this (if any) is quite unknown.

From the foregoing discussion it is evident that no convincing explanation can at present be given of the special significance and constancy of the $9+2$ pattern. Of the three possible answers considered, the second seems as probable as any, but no very direct and positive evidence can be produced in its favour.

Finally, it is interesting to note that such modifications of the $9+2$ pattern as have been discovered (ostracod sperm, mammalian sperm) are solely among flagella. (It might, for example, be expected that there would evolve among cilia a concentration of fibres on the side of the effective stroke, since the power of ciliary reversal does not seem in any case to have been very widely preserved.) The ostracod fibre pattern represents a completely new method of producing a vibratile process in a group where $9+2$ cilia and flagella have disappeared. The fibre pattern of mammalian sperm can only be regarded as a device for concentrating contractile effort on one diameter—the vertical diameter in Text-fig. 3*b*. As pointed out above (p. 314) such a pattern is a logical development for a flagellum generating predominantly two-dimensional waves by mechanism 2 (*a*) of the preceding section. In discussing mammalian sperm it has been assumed that thicker fibres can develop greater tension than thinner fibres. It is also conceivable that differences in thickness might lead to differences in propagation rate, and since there are fibres of at least three different thicknesses in the two rings of 9 this would greatly complicate the generation of waves. It is possible, therefore, that the function of conducting impulses from the proximal centriole along the flagellum has been deputed to the inner 9 fibres, which appear to be of equal thickness and would transmit excitation outward at every level to their thicker, outer partners, with which they lie in

close contact in the mid-piece as well as the tail. Certainly any contraction in the inner 9 would be very insignificant compared with that produced by the outer fibres, which are both much thicker and operating at a greater mechanical advantage. While these profound modifications have been in progress in the mammalian sperm *flagellum*, the fibre pattern of the mammalian *cilium* appears to have remained quite unchanged, though there may well be some new feature in the remarkable basal corpuscles of mammalian ciliated epithelia described by Fawcett & Porter.

SUMMARY

1. Cilia and flagella from very diverse sources have now been shown to contain 11 straight fibres—a cylinder of 9 approximately equidistant, outer fibres (250–500 Å. in diameter) surrounding 2 central fibres whose diameter is usually between one-half and two-thirds that of the outer fibres.

2. This paper describes original observations on the cilia of a mussel (*Mytilus edulis*) and a comb-jelly (*Pleurobrachia pileus*); and on sperm tails of sea urchins (*Echinus* and *Psammechinus*), a fresh-water flea (*Notodromas* —Ostracoda), a frog, and various mammals, particularly bull, ram and boar.

3. In *Mytilus* laterofrontal cilia the 9 peripheral fibres join at their bases and end in a basal plate, which is separated by about 250Å. from the basal corpuscle—a cylindrical body about 0·3μ long from which a pair of ciliary rootlets pass down into the cytoplasm. The central pair end 400 Å. from the basal plate. The direction of beat coincides with the axis of symmetry and is at right angles to the line joining the central pair; the effective stroke appears to be in the direction of the odd member of the outer ring, which is bisected by the axis of symmetry.

4. In *Pleurobrachia* comb-cilia the fibre pattern is as in *Mytilus* cilia, but its relation to direction of beat has not yet been ascertained. The 9 outer fibres join at their bases, form a basal ring and then merge gradually, without a break, into the single cross-striated root, which at about 1μ from the cell membrane breaks up into many fine rootlets.

5. The tail of a sea-urchin sperm provides a typical example of a 9+2 flagellum. As has been found in other sperm tails, the central pair are more susceptible to physical and chemical disruption than the outer 9 and presumably differ from them chemically. The mode of attachment of the fibres to the centriole at their base has not been elucidated.

6. The tails of certain fresh-water ostracod sperm contain an arrangement of 6 helical strands (probably 2 contractile and 4 mitochondrial) which is quite different from the 9+2 pattern.

7. Mammalian sperm tails are also atypical, but less so than those of ostracods. They have a fibre pattern (9+9+2) which is clearly an elaboration on the 9+2 theme. Their 9 outer fibres are unusually thick in the midpiece, 3 of these (2 on one side and one opposite) being about 1000 Å. thick and 6 about 700 Å. thick. The line joining the central pair is at right angles to the diameter on or near which lie these 3 thickest fibres. In the tail, outer and inner fibres are closely united and of nearly the same diameter (180 Å.). The tail fibres are surrounded by the tail helix. Both the helix and the axial fibres are completely dissolved by pepsin and trypsin. From its other solubility properties, there is reason to think that the helix is non-contractile.

8. The basic apparatus possessed in common by all cilia and flagella is a bundle of straight fibres (with matrix and membrane) plus some kind of basal body. Other structures associated with various 9+2 cilia and flagella —such as helical strands, mitochondria, rootlets and other cytoplasmic fibres—are not directly concerned in producing movement.

9. Possible mechanisms of ciliary and flagellar movement are discussed on the basis of various assumptions, the most important of which are as follows: that the outer 9 fibres are contractile; that the central pair are, in cilia at least, specialized for conduction; and that rhythmical impulses arise at one point in the basal body and circulate round it producing propagated localized contractions in each of the 9 outer fibres.

10. In attempting to explain the constancy of the fibre pattern, it seems impossible at present to show that the 9+2 array is inevitable for reasons connected with mechanics or fibrogenesis. An alternative view is that the 9+2 pattern is nothing more than one of several possible arrangements, which has arisen by chance and become a constant cytological feature because successful mutations in the centrosome-basal body system are very rare.

I am much indebted to the Nuffield Foundation and the Agricultural Research Council for support during this work.

Addendum. During further work in collaboration with Lord Rothschild, a striking 9+2 pattern has been observed in bee sperm. In addition to the 9+2 fibre bundle, there are (as in the sperm of numerous insects) two other strands, one of which shows marked cross banding. Longitudinal sections show no evidence of a helix round the 9+2 fibre bundle, however, so that such a helix can still be regarded as a distinguishing character of mammalian sperm tails.

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EXPLANATION OF PLATES

Note regarding methods. Sections were cut from material fixed for 1–2 hr. in Palade's buffered osmium tetroxide (1 %; pH 7·4), embedded in a mixture of butyl and methyl methacrylates (3 butyl : 1 methyl). They were photographed in an early model Siemens electron microscope at 70 kV. without previous removal of methacrylate. For marine organisms fixative was made up in sea water. *Notodromas* sperm were fixed in 2 % formaldehyde in distilled water. The mammalian sperm in Figs. 21 and 22 were unfixed.

Orientation. The approximate orientation of the sections in Figs. 7, 8*a*, 9, 10, 11 and 12 are indicated in Text-figs. 1*a* and *b*. The plane of each figure is through the broken line labelled with the same number and at right angles to the paper; except in the case of Fig. 7 which is at approximately 45° to the paper.

PLATE 1

Fig. 7. *Mytilus.* Longitudinal section at about 45° to the direction of beat of the laterofrontal cilia showing a metachronal wave, transverse to the direction of beat, preserved in the sectioned material. Cilia on the left are at the end of the effective stroke. Those in the centre and on the right are in various stages of recovery. Arrows indicate cell junctions and *r* indicates cross-over rootlets, as seen in Text-fig. 1*c*.

Fig. 8. (*a*) Oblique section through the bases of many cilia and through the apical regions of the cells which bear them. In the upper region are transverse sections of cilia (*c* 1) showing the ring of 9 separate peripheral fibres and the central fibres. Near the cell membrane (*m*) are sections of microvilli (*mv*) and of cilia whose 9 peripheral fibres have fused to form a cylinder (*c* 2). Just at or inside the cell membrane are several sections (*bp*) through the basal plate (or perhaps the top of the basal corpuscle), and inside the cell membrane are numerous sections through basal corpuscles (*bc*) and roots (*r*). (*b*) Transverse sections of unidentified gill cilia in which the 2 central fibres can be more easily distinguished than in (*a*).

PLATE 2

Fig. 9. *Mytilus.* Slightly oblique section through apical region of 4 laterofrontal cells showing 2 rows of ciliary bases in each cell. As the section passes deeper in successive cells (from right to left) each basal corpuscle (*bc*) is replaced by a pair of rootlets (*r*). The latter appear one on each side of the basal corpuscles and are quite distinct from them. A similar sequence is labelled in an adjacent unidentified series of cilia, where the two rootlets in each case are attached to the same side of the ciliary base. *m.* = cell membrane.

Fig. 10. *Mytilus.* Transverse sections of the two rows of laterofrontal cilia (*lf*) from one laterofrontal cell, and oblique sections through the adjacent frontal cilia (*f*). The direction of beat of the laterofrontal cilia is along the row and their effective stroke is toward the frontal cilia, i.e. in the direction indicated by the arrow in the insert showing two cilia at higher magnification. A tracing of the fibre arrangement in these two cilia is given in Text-fig. 1*d*; in spite of some compression during sectioning, it is clear that the direction of beat is at right angles to the line joining the central pair and that the effective stroke is toward the fibre which is bisected by the direction of beat (fibre 1 in Text-fig. 5) and not toward the pair on the opposite side (fibres 5 and 6 in Text-fig. 5).

Fig. 11. *Mytilus.* Longitudinal section approximately through the plane of beat of a row of laterofrontal cilia showing in most cases only one central fibre (indicated by arrows) as is expected if the direction of beat is at right angles to the line joining the central pair.

PLATE 3

Fig. 12*a* and *b*. *Mytilus.* As Fig. 11, but showing in many cases two central fibres (arrows): possible reasons for this are discussed in the text (p. 310); a low-power lens will assist in the observation of these features; *mv* = microvillus, *bp* = basal plate.

Fig. 13. *Pleurobrachia.* Longitudinal section through base of cilia showing cross-striated roots (*r*) breaking up into many smaller rootlets.

Fig. 14. *Echinus* sperm. Frayed tail showing 9 + 2 fibres. (Preparation by Lord Rothschild and Professor A. Tyler.)

Fig. 15. *Psammechinus* sperm. Transverse section of tail showing 9 + 2 fibres.

Fig. 16. *Psammechinus* sperm. Spurious cross, or helical, striations (*s*) in the tail.

PLATE 4

Fig. 17. *Notodromas* sperm. (*a*) Tail sheath with indications of multi-strand helix. (*b*) Frayed tail showing 4 broad strands, possibly of mitochondrial origin, *m*; and two narrow fibres, *f*. Also in the field is a head *h*.

Fig. 18. Frog sperm. (*a*) Transverse section of the tail showing 9 + 2 fibres in approximately the same orientation as in Text-fig. 6. (*b*) Transverse section of mid-piece showing 9 + 2 fibres surrounded by mitochondria, *m*. Orientation uncertain here because 2 central fibres are not well resolved.

Fig. 19. Ram sperm. Transverse section of the mid-piece showing the 9 + 9 + 2 array of fibres. The 2 central fibres can hardly be separated here, but oblique sections show them to be quite distinct. Of the 9 thick outer fibres, 3 (1 at the top and 2 at the bottom) are distinctly thicker than the other 6. The elliptical outline of the mid-piece is due partly to the section being slightly oblique and partly to compression during sectioning. *mh* = mitochondrial helix; *m* = cell membrane.

Fig. 20. (*a*) Ram sperm. Transverse sections of tails showing apparently only 9 fibres surrounding the central pair (the latter can be resolved only in the lower tail). (*b*) Boar sperm. Transverse section of the tail showing that in reality there are still 2 rings of 9 fibres around a central pair, but the inner and outer fibres on each radius are very closely applied to each other. The fibres of the outer ring are much thinner than in the mid-piece, but 3 of them (numbers 1, 5 and 6) are still distinctly thicker than the other 6. This feature is omitted from Text-fig. 3*c*.

The thickened rib or fin which runs down each side of the tail helix is labelled *r*.

Fig. 21. Bull sperm. Mitochondrial helix and tail helix removed (by treatment with N/5 sodium hydroxide, ½ hr., 20° C.) leaving only the bunch of axial fibres. Once exposed, the latter are soon dissolved by the alkali and only occasionally are specimens like this observed. Arrow indicates a pair of tail fibres—one inner and one outer fibre—from the same radius as in Fig. 20*b*, or Text-fig. 3*c*. Nearer the head, in the region of the mid-piece, some of the fibres (presumably those from the outer ring) become much thicker than they are lower down in the tail proper, in accordance with the difference in diameter between the outer fibres in Fig. 19 and the outer fibres in Fig. 20*b* (compare also Text-fig. 3*b* with Text-fig. 3*c*).

Fig. 22. Bull sperm after treatment with 6M urea (3 hr., 20° C.) which leaches out the distal parts of the axial fibres leaving an empty or partially empty helix.

THE ORGANIZATION OF THE MITOTIC APPARATUS

By DANIEL MAZIA
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Mitosis is one of those contrivances of nature that are most difficult to understand in terms of mechanism just because they are so easy to understand in terms of purpose. Granted the end attained—the equal distribution of two sets of chromosomes between two daughter cells—we can hardly imagine a more simple means for achieving this end than the pattern of polarized and co-ordinated movements of chromosomes, centres and fibres that we actually observe. Unfortunately I have nothing constructive to say about how the mitotic centres divide, how they find their way to the poles which they define, or how the chromosomes align themselves so exactly with respect to these poles, although these are the essential features of mitosis considered in terms of what is accomplished. But a good bit can now be said, granted that the above events have taken place, about the mitotic apparatus as a fibrous system, its composition, mode of formation and possible mechanisms of action. If these aspects of the problem of mitosis have proved to be more docile toward experimental analysis, it is partly because they are inherently more simple but also, to no small extent, because here one can draw on the store of experience with the fibrous habits of proteins that is embodied in this Symposium.

THE 'REALITY' OF THE MITOTIC APPARATUS

From the very beginnings of the study of mitosis, so well summarized in Flemming's classic (1882), it has been thought that the chromosomes were engaged in the fibre system that I shall refer to as the mitotic apparatus, and that this fibre system was responsible for the essential manoeuvres of the chromosomes. I shall use the term 'mitotic apparatus' as defined by Mazia & Dan (1952) to refer to 'the ensemble of structures constituting the "chromatic" and "achromatic" figures in the classical descriptions of mitosis'. In almost all cases, the mitotic apparatus includes some version of a mitotic spindle. In many forms, including most animal cells, one also observes mitotic centres at the poles of the spindles, around each of which there is a symmetrical fibre system, the aster.

Our basic information about mitosis is derived, inevitably, from observations of fixed and stained preparations. The notions that spindle fibres and astral rays were 'real' fibres derived from the fact that they were seen as such in fixed preparations. About the turn of the century there existed a great body of scepticism concerning the actual existence of the structures observed by the classical methods, and, indeed, substantial reasons were presented for suspecting that they were artifacts (Fischer, 1899). No structure, including the chromosome itself, escaped doubt, but of all the findings of the cytologist, none were more suspect than the fibrous elements of the spindles and asters. Schrader (1944), in the first edition of his monograph on mitosis, devoted a considerable chapter to the question of the 'reality' of spindle fibres.

In my opinion, this question has now been settled quite apart from the work I shall report below. It seems to me that the recent polarization-microscopic studies, especially those of Inoue (1953), and the phase-contrast studies of Cleveland (1953), on the spindle fibres in *living* dividing cells leave little room for scepticism concerning the existence of the fibrous elements of the mitotic apparatus. The electron microscope can be expected to yield much information about these fibres if they do exist, but, since the technique depends on fixation just as did the classical microscopic technique, it cannot in itself contribute to the question of their reality except in so far as we draw on preconceptions as to 'good' and 'bad' fixation. Rozsa & Wyckoff (1951) failed to find the spindle fibres in thin sections of onion root cell, but they have been observed by Schultz-Larsen (1953) in mouse and human testis and by Selby (1953) in Ehrlich mouse ascites. Only recently has there been sufficient emphasis of the fact that, while the electron microscope has high resolving power, its ability to discriminate small structures of similar density is not great, and very often demands supplementary techniques, either of electron staining or deliberate removal of background material (Porter, 1953).

In some of the earlier discussions of the reality of the spindle fibres, the intermediate possibility was raised that they represented regions of molecular orientation, and that the molecules joined to form a continuous system only under the action of fixatives. This is a question that cannot be answered by optical methods, dealing as it does with intramolecular bonding. It is accessible to experiment once the mitotic apparatus has been isolated for chemical study, and will be dealt with later.

THE ISOLATION OF THE MITOTIC APPARATUS

No one will question the conclusion that an understanding of mitosis and the mitotic apparatus will call on all of our experimental resources. It is obvious, however, that at some point in the analysis it will be necessary to feed in some detailed information concerning the chemical units involved and the chemical interactions between them. The current methods of microscopic cytochemistry might permit a minimum chemical identification (we might say that a given structure contains protein or nucleic acid), but even this is difficult in the case of the mitotic apparatus, which is so intricated in the cytoplasm in which it lies. To obtain any detailed chemical characterization of the mitotic apparatus, it seems necessary to isolate it from the cell in as pure a form and in as large a quantity as is feasible. This can now be accomplished in the case of dividing invertebrate eggs, as was first reported by K. Dan and myself in 1952. The following account will pass over the earlier methods and describe the procedure as it is now used.

Obviously a large population of dividing cells—preferably dividing synchronously—must be used, and this is obtained by working with sea-urchin eggs. Most of the data given in this paper were obtained on material from *Strongylocentrotus purpuratus*. After the eggs have been shed, they are treated with trypsin and chymotrypsin to prevent the formation of a fertilization membrane which otherwise would contaminate the preparations. The eggs are fertilized, and are permitted to develop to the desired stage, usually metaphase, of the first division. Division is then stopped by immersing the eggs in 30% ethanol at $-10°$.

This first step in the isolation deserves some comment. It was chosen as a means of stopping division without denaturing and coagulating the proteins of the cell. Its choice was influenced by the experience of biochemists with other proteins. We have found it to be essential thus far, and consider that it does in fact obviate the denaturation of the proteins. If we permit our preparations to warm even to 0°, the contents of the cells manifestly coagulate and the cytoplasm can no longer be dispersed in the next step of the isolation procedure.

The main step in the isolation is the dispersal of the cytoplasm by what may be termed selective solubilization. Instead of attempting to remove the mitotic apparatus from the cell, we remove the cell from it by treatment with an agent that will disperse or solubilize all other structures. Presumably this depends on the existence in the mitotic apparatus of bonds which are resistant to the solubilizing agent. The class of agents with which we have experimented is the detergents. The earlier and more involved procedure of Mazia & Dan employed the synthetic detergent Duponol

(sodium dodecyl sulphate) and required that the mitotic apparatus first be stabilized selectively by treatment with hydrogen peroxide. Presumably a gentler detergent would discriminate between the mitotic apparatus and other cell constituents without this intermediate step. These desirable properties were found in the natural steroid detergent digitonin. When the cells were transferred directly from the cold alcohol to 1% solution of digitonin and shaken, the cytoplasm, membranes, etc., dispersed and the mitotic apparatus floated free, seemingly intact (Pl. 1, figs. 1, 2). Obviously, the constituents of the mitotic apparatus are bonded in some manner that resists the dispersing action of the detergent.

The fact that digitonin can be employed successfully in this simple procedure is especially welcome because information is available as to its effects on biologically active proteins. The work of Wald and his collaborators (e.g. Wald & Brown, 1952) demonstrates that the visual pigment rhodopsin remains active in digitonin solution, and indeed they study the activity of their entire reconstructed visual system, including alcohol dehydrogenase, in digitonin solution.

By means of this simple procedure, involving stoppage of mitosis in cold ethanol, dispersal of the cytoplasm with digitonin, and separation and washing of the isolated mitotic apparatus by low-speed centrifugation, we can obtain considerable quantities of mitotic apparatus, relatively free from contamination, in water suspension. These suspensions are stable for long periods of time in the cold. The isolation procedure is applicable to all stages of mitosis, from early monastral stages with intact nuclei to telophase stages. The homogeneity of the preparations with respect to mitotic stage may be reasonably good, as the illustrations show.

Some attempts have been made to isolate the mitotic apparatus directly by treating the living cells with digitonin. This can be done (Pl. 1, fig. 3), but it is observed that in most cases the mitotic apparatus dissolves in a short time. Possibly this indicates that the cold alcohol is in fact acting on the mitotic apparatus in some way to stabilize it against the detergent, but an alternative hypothesis is that the alcohol extracts some constituent of the system in the presence of which the mitotic apparatus is unstable. Obviously such a system is latent in the dividing cell, for in the normal course of events the mitotic apparatus is broken down immediately upon completion of division. In the sea-urchin egg, this is only a matter of 10 or 15 min. after chromosome separation has begun.

The stabilization of the mitotic apparatus by extraction with cold alcohol might be analogous to the stabilization of the actomyosin in muscle fibres by Szent-Györgyi's procedure of prolonged extraction with cold glycerol. This question of the stability of the mitotic apparatus under various con-

ditions is of intrinsic interest, because it is probably just as important to the cell to tear down the mitotic apparatus after division as to construct it before division.

THE PHYSICAL CHARACTERIZATION OF THE MITOTIC APPARATUS

The mitotic apparatus isolated from those cells with which work has been done—sea-urchin eggs and eggs of several molluscs (*Spisula* and *Mytilus*)—appears, under the phase-contrast microscope, to have essentially the structure described from fixed preparations. Spindle fibres and astral rays are seen quite clearly (Pl. 2, figs. 1, 2). The chromosomes stand out strongly in phase contrast. The growth in the size of the asters during anaphase, evident in the fixed (Wilson, 1895) and living (Swann, 1952) cells, is represented faithfully in the isolated mitotic apparatus. In the low power view shown in Pl. 1, fig. 2, it is easy to identify those cells that are in anaphase by the larger asters as well as by the position of the chromosomes. Pl. 2, figs. 1 and 2, show this fact, as well as other features of the structure of the isolated mitotic apparatus, in more detail. The isolation procedure makes it possible to make a quantitative analysis of the mitotic apparatus at various stages, and we hope to determine whether the anaphase 'growth' actually represents proportionate incorporation of new material.

In the sea-urchin egg, the mitotic centre takes the form of a rather large clear sphere, and the astral and spindle fibres seem to radiate from the surface of the sphere rather than from a central point. ('Mitotic centre' is a deliberately indefinite term that we shall use until we can sort out the relations of 'centrioles', 'centrosomes', 'centrospheres', etc. Wilson (1925) uses the term 'central apparatus' in the same non-committal way.) In themselves, these observations would support the 'reality' of these elements. The isolation procedure is hardly comparable to cytological fixation. On the contrary, every step is designed to avoid the coagulation of proteins, and if this is permitted, by raising the temperature of the alcohol even to 0° or by treatment at some stage with a cytological fixing agent, the isolation fails. Once coagulated, the cell does not disperse under the action of detergents. While we cannot be sure that our material has not been altered chemically, it is reasonably certain that we have avoided those alterations which are the essence of cytological fixation.

The isolated mitotic apparatus has been subjected to electron-microscopic examination. Miss Patricia Harris has carried out the preparation and electron-microscopic examination. A mass of isolated mitotic apparatus was fixed in neutral buffered osmium tetroxide, embedded in methacrylate

and sectioned. The sections obtained were probably not thinner than 0·1 μ and thus did not permit observation at extreme magnifications. Working with a solid mass of isolated mitotic figures, it was possible to find figures which were cut exactly along a meridian, which made interpretation a little easier.

It seems to me that what is observed (Pl. 2, 3) is best described as a gel with condensed regions of higher orientation corresponding to what appears under the light microscope as spindle fibres and astral rays. The fibres do not have sharp boundaries and their fibrils appear to anastomose with the less condensed material in the background. The condensed and oriented bundles in the spindle run from the chromosomes to the surface of the centrospheres, and the latter appear to have litttle internal structure that has survived the preparation process. Obviously, we are observing here what corresponds to the chromosomal fibres of the cytologist. A view at higher magnification (Pl. 3, fig. 1) shows something of the relation between a chromosome, the fibres attached to it, and the fibres in the background, but unfortunately one can observe nothing corresponding to a centromere. In all the electron micrographs no difference between the astral rays and the chromosomal fibres is apparent.

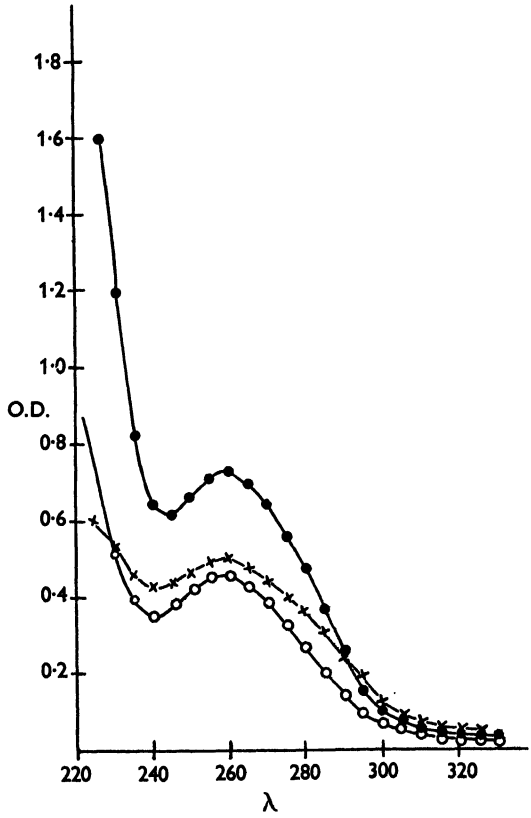
The fibrous elements stand out more clearly in these photographs than they do in most of those that have been published on sections of dividing cells. This is to be expected, since the isolation procedure inevitably improves contrast by removing the cytoplasmic matrix in which the mitotic apparatus is normally embedded.

The interpretation of the mitotic apparatus as a gel whose microscopically visible fibrous elements are regions where the submicroscopic fibrils are condensed and oriented will provide a basis for consideration of the chemical interactions involved in its formation.

CHEMICAL CONSTITUTION OF THE MITOTIC APPARATUS

In order to carry out a chemical analysis of a structure such as the mitotic apparatus it is first necessary to put it into solution. The significance of the solubility studies will be considered in some detail later, since the results bear on the problem of the formation of the mitotic apparatus. At this point it is enough to list the means of putting it into solution: (*a*) trypsin, (*b*) strong alkali (0·5 N-NaOH), (*c*) alkaline thioglycollate (pH 11·5), (*d*) concentrated urea, (*e*) concentrated NaCNS, (*f*) performic acid. Other electrolyte solutions (NaCl, KCl, KBr) fail to dissolve the isolated mitotic apparatus at any concentration. On the contrary, the effect of these

electrolyte solutions is to dehydrate it; it becomes strikingly smaller and more highly refractile. These experiments were of some interest because of the possibility that the behaviour of the proteins of the mitotic apparatus might resemble that of muscle proteins, which is clearly not the case.

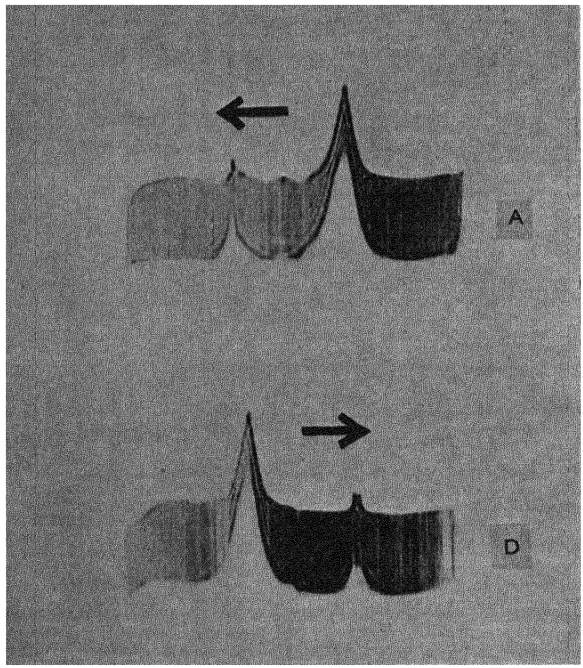


Text-fig. 1. Ultra-violet absorption of the dissolved mitotic apparatus. Upper curve (dots) shows protein precipitable in range pH 5–6, redissolved at pH 7·2. Middle curve (crosses) shows material extractable by cold 5% perchloric acid, evidently RNA or nucleotides. Lower curve (circles) shows material not precipitated in range pH 5–6. To compare the upper and lower curves quantitatively, the optical densities on the latter should be divided by 6 to correct for dilutions. When the correction is made, it is seen that the amount of material in the isolated mitotic apparatus not precipitable in the range pH 5–6 is relatively very small.

In most of our chemical experiments, a solution obtained by dissolving masses of isolated mitotic apparatus in alkaline thioglycollate was used. By analysis, the material was largely protein, containing 2–3% nucleic acid. This is presumed to be RNA because it may be extracted with cold 5% perchloric acid, but it has not yet been subjected to nucleotide analysis. As pointed out in an earlier paper, the contribution of the chromosomal DNA would be quantitatively negligible. The chromosomes of the isolated

mitotic apparatus stain normally for DNA when the Feulgen procedure is applied. It should be mentioned that a qualitative test for polysaccharide, by the Hotchkiss-McManus method, gave positive results, but no analytical work on the carbohydrate content has been done as yet.

If the solution of the mitotic apparatus in alkaline thioglycollate is dialysed against water at about pH 5·5–6, the material precipitates quantitatively (as shown by ultra-violet absorption measurements on the supernatant liquid), often in the form of needle-like aggregates that arouse suspicions of crystallinity, but show little or no birefringence. This precipitate may be dissolved by bringing it to pH 7 or above. An ultra-violet

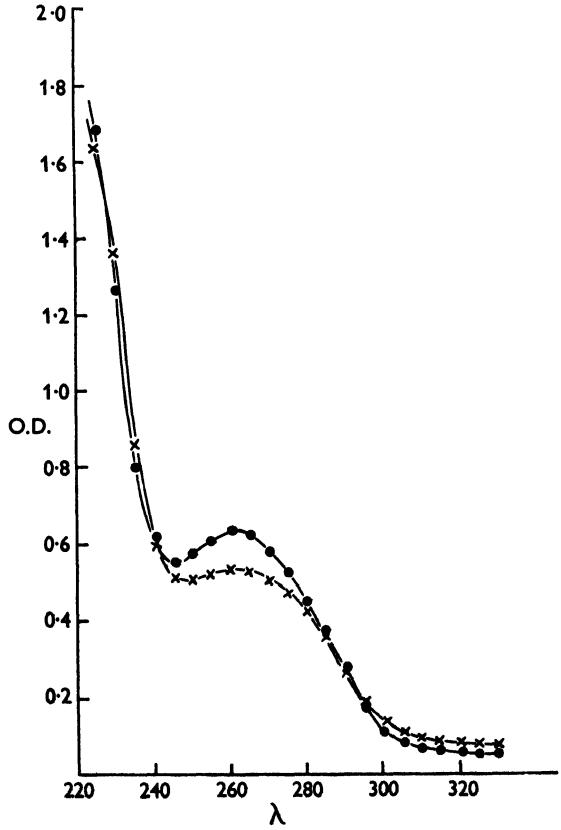


Text-fig. 2. Electrophoretic pattern of the protein of the isolated mitotic apparatus. Phosphate buffer, pH 7·4, 0·02 M with respect to phosphates, 0·15 M with respect to NaCl.

absorption curve of the solution is shown in Text-fig. 1. It is clear that we are dealing with a protein that is relatively low in aromatic amino-acids, containing a small amount of nucleic acid or nucleotides.

The solution of the redissolved mitotic apparatus protein has been subjected to electrophoretic investigation, with the surprising result that it appears to be relatively homogeneous (Text-fig. 2). In addition to the major boundary, there is one small peak which migrates faster. This peak has been permitted to migrate out of the central portion of the cell, and the

remaining material has been recovered for spectrophotometric study. The results are shown in Text-fig. 3. It is quite evident that the fast-moving peak contains most of the material absorbing at 260 mμ, and that the major portion of the isolated protein is a single (in so far as electrophoretic criteria apply) simple protein. Since this pattern represents the total



Text-fig. 3. Ultra-violet absorption spectrum of protein of the dissolved mitotic apparatus before (upper curve) and after (lower curve) electrophoretic separation of the fast minor component. It is evident that the material absorbing at 260 mμ is associated with the fast minor component. pH 7·2.

content of the isolated mitotic apparatus, it seems possible that the bulk of the structure (visibly the spindle and asters) consists of a single protein and that we must look to differences in the orientation of this protein to account for the differentiation into chromosomal fibres, astral rays, and the less well-organized material between the visible fibres. We must remind ourselves at this point, however, that the isolation procedure could have removed some soluble ingredients that may not be essential to the structural integrity of the mitotic apparatus, but could be significant in its function.

The amino-acid composition of the protein making up the bulk of the mitotic apparatus has been analysed and carried out by Mr John Roslansky, using the method of Levy and Chung (Levy, 1954). This method, involving the chromatographic separation of the amino-acids in a hydrolysate after coupling them with dinitrobenzene, permitted quantitative assays on the small samples at our disposal. Some results are given in Table 1. Since considerable attention will be paid to the SH groups in the later discussion, it is of interest that the analysis, calculated in molar proportions, shows the existence of two potential SH groups per weight unit of approximately 20,000.

Table 1. *Relative amino-acid composition of protein of isolated mitotic apparatus*

	Analysis 1	Analysis 2
Cystine	1·0	0·6
Tyrosine	—	1·2
Histidine	—	3·3
Phenylalanine	6·0	6·0
Lysine	9·9	9·3
Proline	8·7	9·6
Arginine	—	9·9
Threonine	10·0	10·0
Alanine	12·0	12·6
Valine	12·0	12·3
Serine	15·8	13·2
Glycine	12·0	14·4
Aspartic acid	20·1	17·7
Glutamic acid	23·1	20·4
Leucine	24·9	23·7

There is, then, reason to believe that in the isolated mitotic apparatus we are dealing with a system that is largely resolvable into protein molecules of a single type. This finding is compatible with the electron-microscopic observations which suggested that the visible fibrous elements were not truly distinct from the gel which forms their matrix, but represent condensed and oriented regions of that gel. If this view is correct, the problem of the formation of the mitotic apparatus is one of assembly and alignment of the molecules of this protein.

THE QUESTION OF THE NUCLEAR ORIGIN OF THE MITOTIC APPARATUS

The proposition that the achromatic elements of the mitotic apparatus originate in the nucleus is encountered commonly in the cytological literature. Cases such as that of the sea-urchin egg, in which the development of the spindle is closely correlated in time with the breakdown of the nuclear membrane (Wilson, 1895), would lend to the assumption of nuclear

origin a certain degree of support. However, now that we can analyse the mitotic apparatus in isolation, it can be shown to be impossible for the nucleus to make a considerable direct contribution to the achromatic figure.

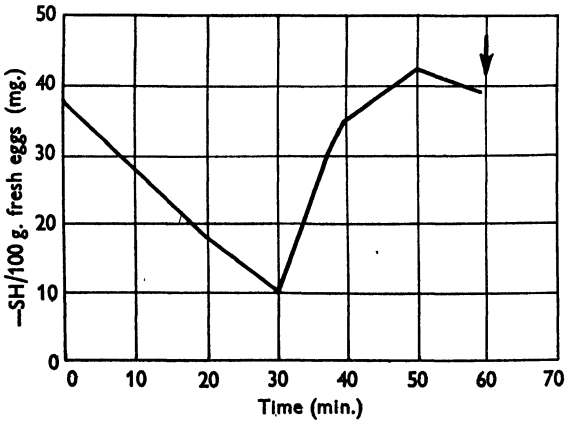
The amount of protein in a single mitotic figure can be determined by direct analysis of a counted sample of isolated mitotic apparatus. The value reported by Mazia & Dan (1952) was of the order of 2×10^{-6} mg. per mitotic apparatus in a sample consisting mostly of metaphases. Roughly, this amount of protein would occupy about 2000 μ^3 when dry. The nucleus of an unfertilized sea-urchin egg has a radius of about 6μ (Harvey, 1932) or a volume of about 900 μ^3. The latter figure represents approximately the maximum amount of protein that the female nucleus could contribute, if it contained no water. But Mitchison & Swann (1953) have estimated the maximum content of dry matter in the sea-urchin egg nucleus to be only 20% of its total weight by the technique of interference microscopy. The total volume of a sea-urchin sperm cell is of the order of a few cubic micra (Hayashi, 1945), so that the sperm nucleus cannot contribute a substantial proportion of the protein of the mitotic apparatus. However important the contributions of the nucleus to the mitotic apparatus may be, it must be concluded that the major part of its protein, and hence of its total mass, is of cytoplasmic origin.

THE ROLE OF S—S BONDS

In exploring the various means of splitting the isolated mitotic apparatus into its component protein molecules, the possibility that they were linked by S—S bonds was an obvious one. Not only the earlier work on keratins but more recent evidence of the role of S—S bonds in gel formation (Huggins, Tapley & Jensen, 1951) would suggest this line of attack. The point of departure was the observation by Goddard & Michaelis (1934) that keratins would be rendered soluble by reduction of their S—S bonds with thioglycollate. They found that alkaline conditions were required, presumably to split the secondary linkages remaining after the S—S was reduced. The same considerations apply to the isolated mitotic apparatus. It is completely dissolved in 1% sodium thioglycollate at pH 11·5. Without the thioglycollate, exposure to alkali at this pH has no effect.

It seems very likely that S—S bonds (or at least bonds involving the oxidized state of S) do play a decisive part in the structural associations of the protein of the mitotic apparatus. A simple proposal would be that one step in the formation of the mitotic apparatus is polymerization of the protein through intermolecular S—S links, and such a proposal recalls the rather extensive evidence that thiol groups are somehow involved in mitosis.

For our purpose, the starting-point is the study of the behaviour of glutathione during mitosis in the sea-urchin egg, published by Louis Rapkine more than 20 years ago (Rapkine, 1931). Measuring the trichloracetic acid-soluble —SH groups, he observed the cycle shown in Text-fig. 4. The concentration of soluble —SH declines during the first two-thirds of the period between fertilization and cleavage. At the time when the minimum —SH concentration is reached the mitotic apparatus in the sea urchin consists of two small asters with the nucleus lying between them, its membrane still present and the chromosomes condensing within it. The soluble —SH concentration now begins to increase, reaching its initial value at about the time the cytoplasm begins to divide. This is the period



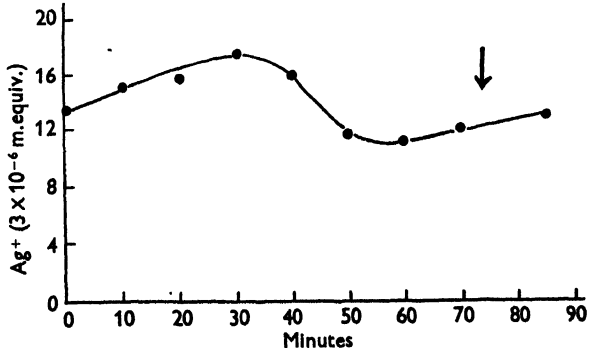
Text-fig. 4. Rapkine's data on variation of soluble —SH (glutathione) during the first division of the sea-urchin egg (*Paracentrotus lividus*). Arrow marks time of cytoplasmic cleavage.

when the mitotic apparatus is forming fully and growing. The nuclear membrane breaks down, the chromosomes condense, the spindle is formed (its fibrous elements being clearly visible even before the alignment of the chromosomes on the equator), and the asters show a remarkable growth (Pl. 2, fig. 3 and Pl. 3, fig. 1) as the chromosomes are moved apart. It can be said that the soluble thiol groups are being oxidized during the preparatory stages of mitosis, and are being reduced during the period of full formation of the mitotic apparatus and its growth.

In view of the widespread relation between soluble —SH groups and enzyme activity, which he had been instrumental in establishing, it was natural for Rapkine to seek the explanation of his results in this direction. While it is entirely conceivable that the fluctuating glutathione concentration may have significant consequences for enzyme activities during mitosis, no such relationship has been found as yet. The possible significance of the

—SH groups for *structural* rather than enzymatic events has been neglected, but now that there is some evidence that S—S bonds are implicated in the assembly of the protein of the mitotic apparatus, we may refer back to the dividing cell and ask whether there is any relation between Rapkine's glutathione cycle and the thiol bonding of proteins.

This can be done by comparing the behaviour of the —SH groups of the proteins with that of soluble —SH. Such an investigation is now being carried out by Mr Gordon Ellis. His method is a version of the amperometric titration introduced by Kolthoff & Harris (1946). He follows the total protein-bound (TCA-insoluble) —SH at intervals during the first division of the sea-urchin egg (*S. purpuratus*). Text-fig. 5 shows the results



Text-fig. 5. Variation of protein-bound SH during the first division of the egg of the sea-urchin *Stronglyocentrotus purpuratus*. Arrow marks time of cytoplasmic cleavage. Ordinates are equivalents of Ag used in amperometric titration of samples containing the same number of eggs. Unpublished data of Gordon W. Ellis.

obtained thus far. Allowing for the difference in the time scale of division in the *Strongylocentrotus* egg and in the *Paracentrotus* egg that Rapkine used (attributable chiefly to the fact that sea-urchin eggs from the California coast will divide only at temperatures less than 20° C.), one sees that the curve for protein —SH is essentially the reverse of Rapkine's curve. The exact stoichiometry has not yet been worked out, but it is evident that the one curve is rising when the other is falling, and both reverse at the same stage of division.

It is therefore possible to propose (subject to a closer quantitative analysis) that there takes place during division in the sea-urchin egg an oxidation-reduction cycle between glutathione and thiol groups in proteins. During the first phase of the cycle, the glutathione is oxidized, reducing protein S—S to —SH. During the second phase, the reverse takes place. The point of reversal corresponds in time to the establishment of the

karyokinetic figure in the classical sense (Wilson, 1895): the formation of the spindle and the incorporation of the chromosomes into the spindle.

How can this cycle, timed so significantly, be interpreted? From the raw chemical data, it would appear merely that a certain number of S—S bonds in the protein was first reduced, then reoxidized. But an alternative interpretation is suggested by some very interesting work from the laboratory of Huggins, cited earlier. This group, investigating the role of S—S bonds in the gelation of proteins *in vitro*, obtained evidence that mercaptide exchanges might play a part in the establishment of intermolecular links. That is, intramolecular S—S bonds may be converted to intermolecular S—S bonds. At the end of the process, the total number of disulphide bonds would be about the same as that at the beginning, but the molecules would now be linked in chains or in a network. Text-fig. 6 reproduces their diagram of a chain mercaptide exchange reaction initiated by an —SH compound and leading to the formation of a gel.

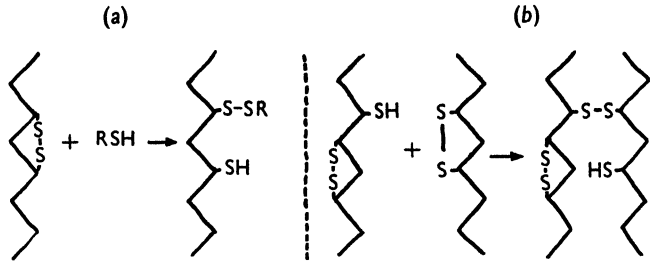
In the case of the formation of the mitotic apparatus, we have considered two schemes that adapt the same principle to the observed oxidation-reduction cycle between soluble —SH and protein —SH. These two schemes are diagrammed in Text-figs. 7 and 8.

A choice between these two schemes—if, indeed, either is valid, will be possible only when our work has progressed to the point where the stoichiometry has been determined. Meanwhile we prefer the second on the basis of work by Infantellina & La Grutta (1948) and by Bolognari (1952). These investigators have dealt with glutathione in sea-urchin eggs and have found that when the concentration of reduced glutathione decreases, there is no corresponding increase in the concentration of the oxidized form. In fact, the concentration of the oxidized form is extremely low at all times. If, as proposed above, the oxidation of glutathione is related to the reduction of protein S—S, then, according to the second scheme, the oxidized glutathione might be carried in the protein fraction, and not be found by the assay method for oxidized glutathione.

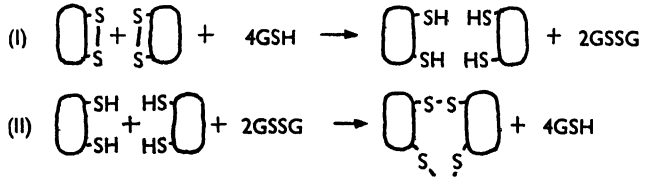
These data do relate at least one aspect of the formation of the mitotic apparatus—the establishment of its S—S bonding—to the events in the cell as a whole. At the same time they pose a question for which I do not have even a tentative answer. This is: what metabolic process in the dividing cell can account for a reversal of the glutathione-protein oxidation-reduction system such that during one phase glutathione serves as a reducing agent and during the second phase as an oxidizing agent?

The aggregation of relatively small protein molecules by intermolecular S—S bonding will obviously produce a system which would tend to have the properties of a gel. Two pictures of the system are possible. The S—S

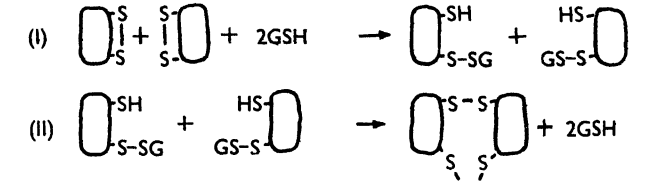
bonding may be essentially one-dimensional, leading to long chains of molecules, or it may be more random, producing a network in which S—S bonds provide cross-links. The rather low content of cysteine in the protein might tend to favour the formation of single chains, since there would be few SH groups available for cross-linking. Whether or not there were an



Text-fig. 6. Mechanism of gelation by mercaptide exchange reactions. (From Huggins, Tapley & Jensen 1951).



Text-fig. 7. Possible mechanism of polymerization of protein of the mitotic apparatus through oxidation-reduction cycles of glutathione and protein. Essentially, intramolecular S—S is converted to intermolecular S—S. According to this mechanism, oxidized glutathione should be found during the first part of the cycle.



Text-fig. 8. An alternative mechanism of polymerization of the protein of the mitotic apparatus. Oxidized glutathione would not be detected.

appreciable number of S—S bonds cross-linking the molecular chains, the whole system would tend to have the properties of a gel if the chains were sufficiently asymmetric.

An experiment has recently been suggested to me by Dr H. Fraenkel-Conrat which would permit a closer examination of the position of the S—S bonds. As mentioned earlier, the mitotic apparatus is soluble in strong urea, which is not the case for keratins which are strongly cross-linked by S—S bonds. This in itself suggests that other types of bonds (one thinks

first of hydrogen bonds) are essential to the structural integrity of the mitotic apparatus. As mentioned, it is conceivable that the S—S bonds are used primarily to form chains of the small protein molecules of which the mitotic apparatus is ultimately composed. If such chains exist, and are further associated by secondary bonds, then they should be set free by solution in urea, which would not be expected to affect the S—S bonds. Results obtained thus far conform to this prediction. When a suspension of mitotic apparatus is dissolved in 6–10M urea, the resulting solution is highly viscous. Solutions obtained with the aid of thioglycollate are not viscous. Therefore, urea dissociates the mitotic apparatus into linear aggregates which, it is proposed, are the S—S bonded chains of the protein molecules making up the bulk of the mitotic apparatus. Conversely, S—S bonding cannot contribute very much to the lateral associations of these molecular fibrils.

SECONDARY BONDING AND THE ROLE OF THE MITOTIC CENTRES AND THE CENTROMERES

The processes I have been discussing—polymerization by S—S bonds—need only establish a gel of some kind, and in themselves do not account for the symmetry and orientation observed in the mitotic apparatus. A possible clue has developed, however, from a closer investigation of what I shall call secondary bonds in the assembly of the mitotic apparatus: bonds other than S—S bonds. The evidence for the existence of these was (*a*) solubility in urea, (*b*) the fact that thioglycollate dissolves the mitotic apparatus only under alkaline conditions. Work on keratins (Jones & Meecham, 1943) has shown that after reduction of S—S bonds, the secondary bonds remain, but can be split at high pH as well as by detergents and other 'dispersing agents'. The solubility of the mitotic apparatus in 1% thioglycollate at various pH values has been observed. Up to pH 10·5, the apparatus swells but does not dissolve; upon return to neutral pH its appearance becomes normal except for the absence of the chromosomes. Above pH 10·5, dissolution is progressive with increasing pH. The equatorial region and the peripheral parts of the asters dissolve as the pH exceeds 10·5 and the solution progresses toward the mitotic centres as the pH is raised until at pH 11·0 only the centres, chromosomal fibres and the portion of the aster closest to the centrospheres survive (Pl. 3, fig. 2).

If we may suppose that the regions which have the highest concentration of alkali-sensitive bonds require the highest OH concentration for the dispersal of these bonds, it would be concluded that these bonds are most concentrated near the centres and that their concentration falls off with

distance from the centres. How shall such a gradient be interpreted? An obvious assumption would be that the gradient is the result of outward diffusion of some bonding agent from the centres. If the concentration of bonds is related to the concentration of bonding agent, then the observed results would be predicted. Moreover, such an hypothesis would account for the symmetry of the mitotic apparatus. If the unit fibrils were laterally associated and condensed into microscopic fibres by the action of an agent emanating from the two centres, one would expect just such a picture of two fibrous asters with a fibrous spindle between them. Conversely, the finding that the microscopic fibres have the same composition as the gel in which they lie, differing only in condensation and orientation, would make sense.

If this rather general hypothesis has any merit, it would represent the closing of a circle in the history of the subject. We may recall the work of Fischer (1899), who was among the first to examine the validity of cytological methods of fixation and staining. He reasoned that any structure they could produce as an artifact in a model system might be an artifact in a cytological preparation. Fischer carefully introduced small drops of fixative solutions into protein solutions. The result was the precipitation of the protein in star-shaped figures resembling those seen in mitotic figures. If the mechanism of formation of the mitotic apparatus proposed above has any validity, Fischer was not merely studying the genesis of artifacts, but was setting up a crude model of the way the fibrous pattern actually may be laid down in the cell!

A further postulate is necessary, namely, that the regions of spindle-fibre attachment on the chromosomes (centromeres of kinetochores) are acting in the same manner as the mitotic centre. That is, they must also be sending into the gel a secondary bonding agent similar to that coming in from the centres. The combined action of the two would be responsible for the fibres connecting the chromosomes to the centres. One part of this hypothesis is in harmony with the findings of cytologists who have investigated the problem (e.g. Pollister, 1939): there is considerable evidence that the centromeres and the mitotic centres are homologous structures. Therefore, if the function of the centres is to produce a secondary bonding agent, it is not far-fetched to suppose that the centromeres perform the same function. Some of our experiences with the isolation of the mitotic apparatus led to the conclusion that the chromosomal fibres are more strongly bonded than the astral rays or other components of the system. I have already referred to the necessity for extraction with cold 30% ethanol as a preliminary step in the isolation of the mitotic apparatus. If the extraction is incomplete, then subsequent application of digitonin results in the dissolution of the astral rays and of the spindle components other than the chromosomal

fibres. Centres connected to the chromosomes by chromosomal fibres remain. This result would be anticipated if the chromosomal fibres are reinforced by the action of the chromosomes.

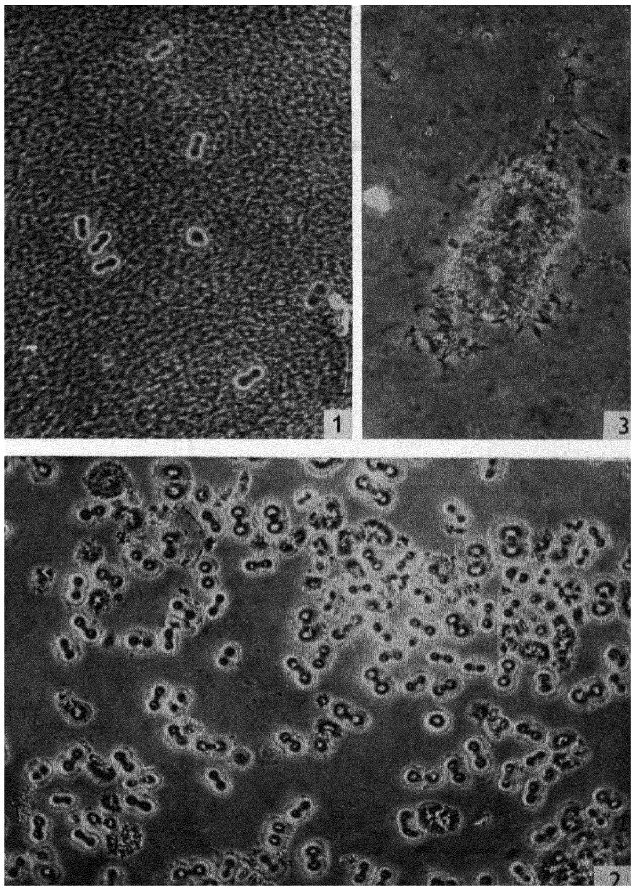
Since we have no evidence concerning the nature of the supposed secondary bonding agent, the question whether the formation of the mitotic apparatus actually involves two mechanisms of molecular interaction—S—S bonding to form the basic gel and lateral bonding to establish the fibrous pattern observed with the light microscope—can best be resolved by experimental dissociation of the two processes. This has been accomplished through the study of mitotic inhibition.

THE MECHANISM OF ACTION OF COLCHICINE

Colchicine is the archetype of those antimitotic agents which are believed to inhibit the mitotic spindle. In 'colchicine mitosis' the chromosomes typically go to metaphase, but are not separated. In the massive literature on the subject it is not always clear whether colchicine is thought to prevent the formation of the spindle or to block its motor functions.

Mr John Roslansky, in our laboratory, has applied the isolation method to sea-urchin eggs in which mitosis was blocked at metaphase by the application of colchicine shortly after fertilization. The argument is that if colchicine actually prevents the formation of the spindle, no spindle can be isolated, but if it creates some abnormality, that abnormality may be studied best in isolation. The result was that a mitotic apparatus, containing chromosomes in or approaching metaphase alignment, could be isolated, but what was recovered was an amorphous mass of gel showing no microscopic fibres (Pl. 3, fig. 3)! We conclude from these observations that the formation of the mitotic apparatus may indeed be separable into two processes, a polymerization process giving rise to a shapeless gel (which might not be detected by cytological methods and which would lack birefringence), and a second process condensing and orienting the elements of the gel into the fibre system observed in the normal mitotic apparatus. In terms of the hypothesis of the formation of the mitotic apparatus discussed above, one would say that the colchicine has not affected the process leading to the formation of intermolecular S—S bonds, but has inhibited the secondary bonding processes that were attributed to the action of the mitotic centres and the centromere regions of the chromosomes. This interpretation presents interesting possibilities for further work on the mechanism of action of antimitotic agents. It excludes the notion that agents like colchicine completely inhibit the process of formation of the spindle. It lends no encouragement to the approach whereby colchicine

PLATE I



For explanation see p. 357

(*Facing p.* 352)

PLATE 2

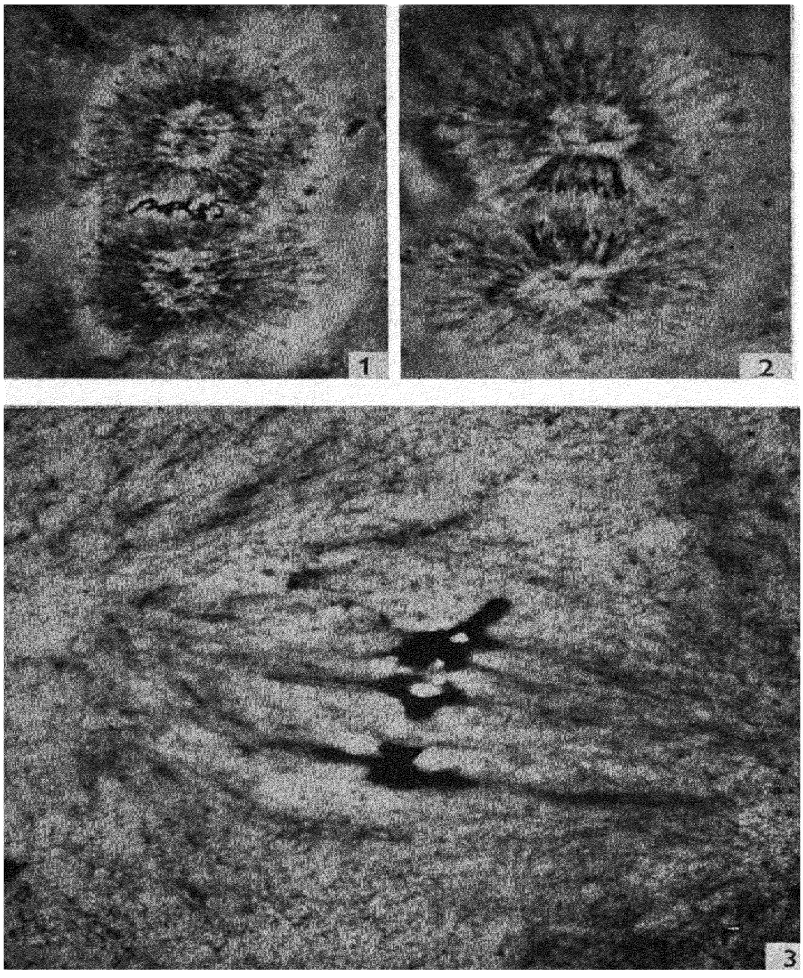


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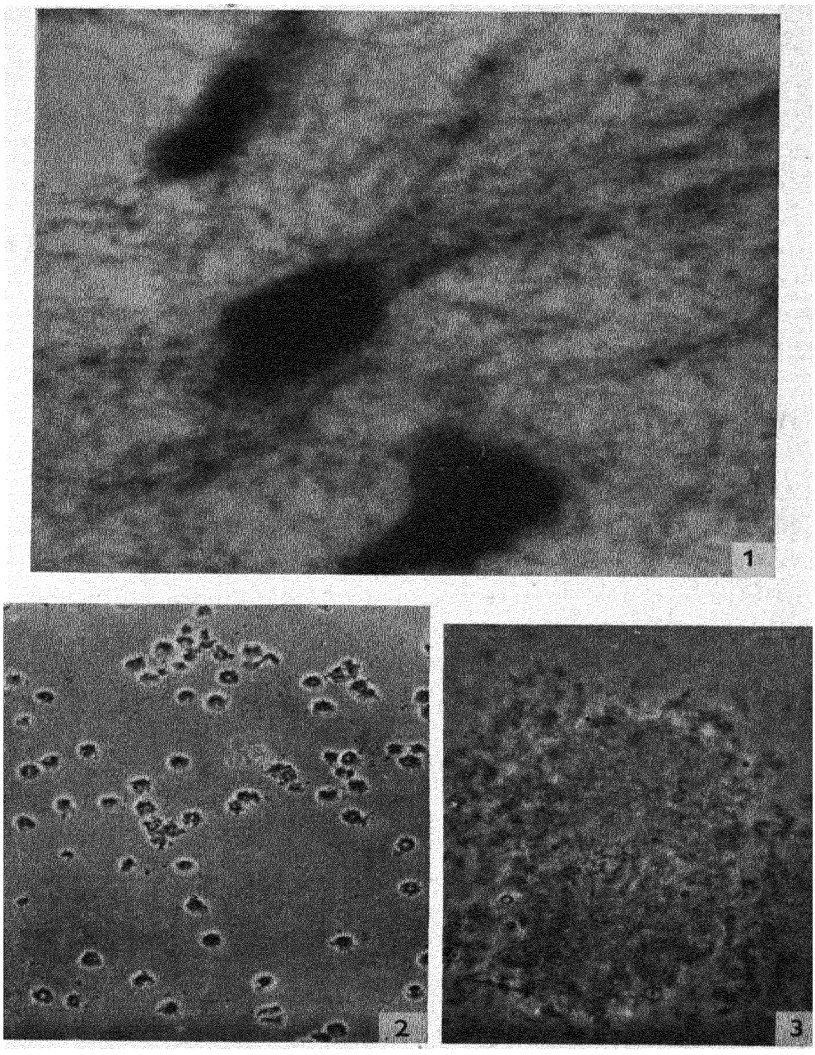
PLATE 3



For explanation see p. 357

is considered to be a possible inhibitor of energy-yielding processes underlying mitosis. On the other extreme, it does not favour the idea (Lettre, 1952) that colchicine may affect contractile functions as such. Rather, it tends to focus attention on one step in the formation of the complete mitotic apparatus, and relates that step to structures—centre and centromeres—whose function has been unknown. However, for the present time, this experiment is of interest in that it permits us to dissociate the formation of what Heilbrunn (1953) has called the mitotic gel from the establishment of the order fibrous organization of that gel.

FUNCTION OF THE MITOTIC APPARATUS

While the study of the isolated mitotic apparatus promises to give some insight into the manner of its formation and the relation of its formation to other events in the dividing cell, thus far we have been unsuccessful in attempts to put it to work. From the beginnings of the history of the subject the possibility that the chromosomes were pulled by a system analogous to muscle has been the subject of speculation. Dr Weber, in this Symposium, will present recent evidence to this effect. Thus far our attempts to press the analogy in the case of the isolated mitotic apparatus have been unsuccessful. It can be said that the proteins are not soluble under the conditions for solution of myosin. In fact, the isolated mitotic apparatus shrinks at high salt concentrations. Application of ATP-electrolyte mixtures has not yielded any changes other than the over-all dehydration obtained with electrolytes alone. Preliminary tests for ATPase activity have been negative. Needless to say, such negative results might only mean that the isolation procedure has brought about denaturation of the system, but they could also mean that it is different from the only contractile system that has been studied in any detail—skeletal muscle—in important respects.

There is one aspect of our findings for which the muscle analogy is conceptually useful. It may well be asked why, if the bulk of mitotic apparatus is chemically as homogeneous as it appears to be, the chromosomal fibres are the only elements which undergo visible contraction during mitosis. The observations on muscle models (Szent-Gyorgyi, 1950; Hayashi, 1952) suggest that the ability of actomyosin fibres to contract under a load is a function of the degree of intermolecular cross-linking. If the same considerations apply to the mitotic apparatus, and it is formed according to the mechanism proposed in this paper, then only the more strongly cross-linked regions would be expected to contract against resistance.

SUMMARY

The progress of the studies I have described has permitted the fabrication of a picture of how the mitotic apparatus is formed in the dividing cell and the interpretation of certain persistent problems of cytology in terms a little more concrete than has seemed possible on the basis of microscopic observation alone. Past experience shows that when a 'story' first emerges from a collection of observations, a situation soon arises in which interpolations that provide the plot are confused with the proved facts. I propose, therefore, in summarizing this report, to evaluate the factual basis of the ingredient items.

(1) The mitotic apparatus may be isolated, by methods that minimize protein coagulation, as a unified entity that includes all of the structures, chromatic and achromatic, recognized in the classical picture of mitosis. The isolation procedure is valid for any stage of the cycle. By morphological criteria this is a fact; every structure that one looks for is observable in our isolated preparations. It is not proved, however, that the isolated mitotic apparatus is chemically intact. In the living cell it may contain important chemical constituents which are removed by our isolation procedure.

(2) On the basis of electron-microscopic observation, the isolated mitotic apparatus can be described as a gel containing regions where fibrils are condensed and oriented to form larger fibres. The chromosomal fibres and astral rays appear to be the result of such condensation and orientation. This describes the pictures obtained. Once isolated, the mitotic apparatuses were processed for electron microscopy by methods now generally considered to minimize artifact. We cannot, obviously, be certain that the preparations are free from artifact, and it is certain that technical improvement will permit observation of finer detail.

(3) The mitotic apparatus in the metaphase of the first cleavage of the sea-urchin egg accounts for about 2% of all the protein in the egg. When dissolved in alkaline thioglycollate, it gives a solution of protein containing a small amount of RNA. The presence of DNA in the chromosomes of the isolated apparatus is shown by staining, but it is quantitatively inconsiderable. Adequate analyses for lipids or carbohydrates have not been made. These are facts.

(4) The dissolved mitotic apparatus, subjected to electrophoretic analysis, shows two components: a simple protein accounting for most of the material and a nucleoprotein present in much smaller amounts. It is supposed, in the subsequent interpretation, that the formation of the fibrous spindle and asters is largely accounted for by the behaviour of the predominant simple protein. The function of the ribonucleoprotein is not

considered, although the results of staining and ultra-violet experiments of other authors suggests that it behaves in a regular fashion (Jacobson & Webb, 1952; Walker & Yates, 1952). The major components have been subjected to amino-acid analysis which suggests a minimum molecular weight in the neighbourhood of 20,000.

(5) Solution of the mitotic apparatus in strong urea gives rise to a viscous solution. This has been interpreted as signifying that the molecules described above are associated end to end by bonds not split by urea, and laterally by bonds that are split by urea. This is inference; the detailed properties of the proposed molecular chains have not been studied.

(6) The formation of intermolecular S—S linkages has been related to the oxidation-reduction cycle between glutathione and protein in the dividing egg. Rapkine's data show a cycle of glutathione concentration with a minimum just before the time of spindle formation. Data from our own laboratory indicate that the protein —SH varies reciprocally to the glutathione. These are preliminary observations: Establishment of the oxidation-reduction relation as a fact will depend on more refined quantitation of the behaviour of glutathione and protein SH. On the basis of the preliminary facts, a model of the mechanism of the formation of S—S bonded chains has been proposed, employing a cycle which converts intramolecular protein S—S to intermolecular S—S.

(7) The mass of S—S bonded protein chains would give an unoriented gel, chemically homogeneous. Evidence from solubility experiments suggests that the fibrils of the gel may be condensed laterally by means of secondary bonds, giving oriented clusters which account for the microscopic fibres. Suggestive but not conclusive experiments suggest that the concentration of the secondary bonds decreases along a gradient extending outward from the mitotic centres. Other evidence, also not conclusive, suggests that the chromosomes are contributing to the secondary bonding. It is proposed as an hypothesis that the secondary bonding, and hence the geometric pattern of fibres in the mitotic apparatus, is determined by the diffusion of an agent from the mitotic centres and from the centromeres of the chromosomes.

(8) The dual mechanism of the formation of the mitotic apparatus—but not any details of the mechanism—is demonstrated by the study of the action of colchicine. The mitotic apparatus of the colchicine-blocked sea-urchin egg may be isolated as an amorphous gel, without evidence of microscopic fibres. An hypothesis of the mechanism of action of colchicine is that it affects the secondary bonding action attributed to the mitotic centres and to the centromeres.

There is no need to list all the unsolved problems generated by this analysis. It has been made evident that the study of the chemistry and formation of the mitotic apparatus has not yet given us any insight into its action, except to expose a few points at which the analogy to muscle breaks down. One question that is raised is whether the protein used to form the mitotic apparatus pre-exists as such before division, or whether it is synthesized or derived from modification of some pre-existing protein. Recent work on blood-clotting opens up the latter possibility. A second is the significance of the ribonucleoprotein which we have found and separated from the main mass of protein. Finally, once the student of mitosis has gained any insight into the mode of formation of the mitotic apparatus, he will inquire into the mechanism of its breakdown after mitosis is complete. For this working mechanism, unlike muscle or any other motor mechanism one can think of, is formed anew by the cell each time it is used, and disappears from view when its task is done.

The question arises inevitably, whether these studies of the chemistry of mitosis in one type of cell apply to other types. In particular, their applicability to plant cells in which centres and asters have not been observed by cytological methods, will be questioned. It seems to me that these questions are not entirely legitimate because they impute a greater significance to appearance alone than may be justified. If, for instance, we can obtain evidence that the polar regions of the dividing plant cell have solubility properties comparable to those of the mitotic centres of the sea-urchin egg, and may equally well be presumed to be the source of a secondary bonding agent, such an observation would tend to unify the mechanism of mitosis in the two cell types regardless of whether or not we can stain a definite granule and call it a centriole. Once the study of mitosis has been pushed beyond the descriptive stage, the search for underlying unity becomes the most interesting of the future prospects.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. First step in the isolation of the mitotic apparatus. Eggs have been extracted in 30% ethanol at −10° and shaken in 1% digitonin. Dispersed cytoplasm in background. Mitotic figures suspended in granule suspension. Phase contrast (× 100).

Fig. 2. Purified preparation of isolated mitotic apparatus, separated from cytoplasmic granules by differential centrifugation. Larger figures (one indicated by arrow) are anaphases and telophases, illustrating growth of aster during this period. Phase-contrast view (× 100) of water suspension.

Fig. 3. Mitotic apparatus isolated directly from living cell by treatment with digitonin. Most of the mitotic apparatuses dissolve, but those that may be recovered by rapid removal of digitonin appear normal, indicating that pre-extraction with cold alcohol does not produce visible alterations. Phase contrast (× 400).

PLATE 2

Fig. 1. Isolated metaphase at higher magnification (× 900).

Fig. 2. Isolated anaphase at higher magnification (× 900).

Fig. 3. Electron micrograph of thin section of mitotic apparatus at metaphase, illustrating gel structure (× 5400).

PLATE 3

Fig. 1. Higher-power electron micrograph of a chromosome and associated fibres. Note how these anastomose with fibres in background (× 29,100).

Fig. 2. Action of 1% thioglycollate at pH 11·0. Only the mitotic centres and the fibres closest to the centres remain.

Fig. 3. Mitotic apparatus of colchicine-blocked egg (× 900).

PROBLEMS OF STRUCTURE AND FUNCTION IN THE AMPHIBIAN OOCYTE NUCLEUS*

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INTRODUCTION

A curious failing of research in cellular physiology up to the present time has been its inability to assign specific roles to the nucleus in ordinary metabolic processes. The old view of the nucleus as the oxidative centre of the cell has fallen into complete disrepute with the demonstration of respiratory enzymes on the mitochondria. Likewise theories attributing the 'general synthetic properties' of the cell to the nucleus fail in the light of enucleation experiments and of recent studies on enzyme distribution (cf. the discussion in Mazia, 1952). At present many workers incline to the view that the nucleus is a site of protein synthesis or in some indirect way controls the specificity of proteins elaborated elsewhere. Although an understanding of the mechanisms of protein synthesis must be based ultimately on chemical methods, nevertheless a study of morphological relationships within growing cells can lead to fruitful suggestions regarding the localization of activity. Thus without answering the question of whether the nucleus is the *major* site of protein synthesis, we can easily find nuclei in which a demonstrable accumulation of protein is taking place. These cases can be studied for information on the origin of the accumulated protein and hence yield insight into the role of the nucleus in its formation. The present paper will consider one such nucleus, the amphibian germinal vesicle, in an attempt to formulate a plausible scheme of nuclear protein synthesis during oogenesis.

Growth of the vertebrate oocyte nucleus has been described many times (e.g. Rückert, 1892; Duryee, 1950; Callan, 1952; Guyénot & Danon, 1953). The general outline will be reviewed here by way of orientation, using the salamander *Triturus viridescens* as an example. Almost all the observations discussed later in the paper were made on this form; where specific reference to various points is not given, the original data will be found in the paper by Gall (1954*a*). The oocyte begins development in the ovarian wall as a relatively unspecialized cell approximately 25–30 μ in diameter with a very large nucleus. The early phases of meiosis (leptotene, zygotene, pachytene)

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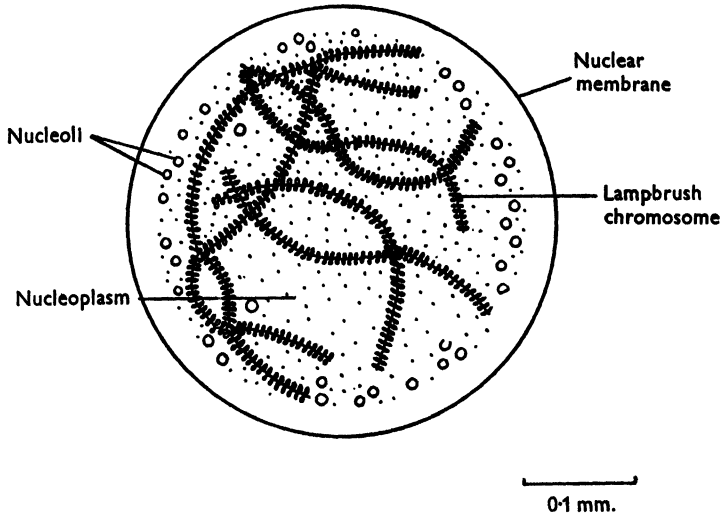
occur before any major changes in size take place. However, at diplotene the usual course of meiosis is stopped and growth of the oocyte begins. The cytoplasm increases in quantity, mitochondria appear in large numbers, and eventually yolk deposition begins (Wittek, 1952; Panijel, 1951). At the same time the nucleus itself enlarges, maintaining an almost constant nucleus/cytoplasm volume ratio (about 1:7) until the egg reaches half maximum diameter; after this point the nucleus grows only slightly. The chromosomes likewise enlarge, developing eventually the typical 'lampbrush' condition characterized by extreme length (up to 800 μ) and by the production of hundreds of loop-like projections extending laterally 10–15 μ



Text-fig. 1. Diagram of the nucleus from a medium-size oocyte. Later the chromosomes contract to form a small core in the centre of the nucleus.

or more from the main chromosomal axis. Large numbers of nucleoli accumulate just under the nuclear membrane during this period of growth. At its greatest enlargement the nucleus may attain a diameter of over 400 μ, so that its overall volume increase during oogenesis is about 8000 times.

Much of the accumulated material is of course protein (evidence reviewed later). Let us begin our inquiry into the origin of this protein by examining the relative volumes occupied by the various parts of the nucleus (Text-fig. 1).

(*a*) Very roughly the nuclear volume is $\frac{4}{3}\pi\,(200)^3$ or about $320 \times 10^5\,\mu^3$.

(*b*) There are eleven pairs of chromosomes in *T. viridescens* whose total length is nearly 6000 μ at maximal extension. Considered as solid cylinders of diameter 20 μ (a gross overestimate assuming the lateral loops a solid mass), they have a total volume of 2π 6000 $(10)^2$ or about $36 \times 10^5\,\mu^3$.

(*c*) Finally, there are about 1000 nucleoli, roughly spherical, with a mean diameter in the vicinity of 10μ, giving a total nucleolar volume of $1000 \times \frac{4}{3}\pi\,(5)^3$ or $5 \times 10^5 \mu^3$.

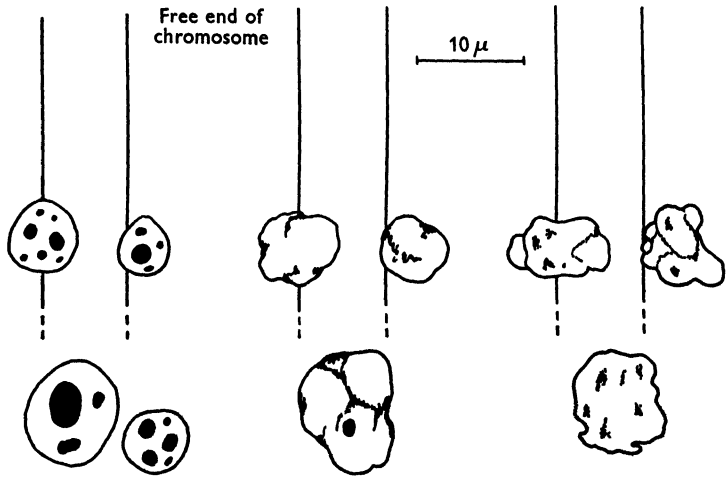
These values are only approximations but they do illustrate an important point: at least 90% of the nuclear volume, and probably more, is occupied by extra-chromosomal material even at the maximal enlargement of the chromosomes. Furthermore, although the nucleoli are the cytologically most conspicuous extra-chromosomal elements, their total volume relative to that of the nucleoplasm is quite small. Unless therefore the nucleoplasm is only an 'inert' bathing fluid, its origin is likely to be of as much interest with respect to protein synthesis as the origin of the chromosomal loops or of the nucleoli. However, the nucleoli form the most convenient starting point for an examination of the synthetic activity of the nucleus, since their characteristic morphology permits a close study of their formation. Furthermore, nucleoli have played a predominant role in theories of intracellular protein synthesis (e.g. Caspersson, 1950; Brachet, 1952*a*, *b*).

NUCLEOLI

In general, a full-size nucleus, or one near its maximum size, contains 1000 or more refractile, extra-chromosomal nucleoli, ranging from less than 10 to nearly 20μ in diameter. Although usually somewhat spherical the nucleoli present a bewildering array of morphological aspects in different nuclei. The majority are situated just beneath the nuclear membrane with a few scattered elsewhere in the nucleoplasm. Amphibian oocyte nucleoli are composed largely of protein (solubility experiments of Gersch, 1940), but contain easily demonstrable quantities of ribonucleic acid (RNA). Comparable data are available from analyses of isolated nucleoli from starfish oocytes (Vincent, 1952), which show a predominance of phosphoprotein in association with RNA, and in addition small amounts of lipid and soluble nucleotide.

The oocyte nucleoli are commonly said to form on the chromosomes, but the implications of this statement vary considerably from one author to the next. Confusion arises from the fact that hundreds of granules of various sizes are attached all along the chromosomes. That these granules, for the most part considerably smaller than the peripheral nucleoli, detach from the chromosomes and subsequently grow into full-size nucleoli has usually been a tacit assumption. On the other hand, several workers have stated that the nucleoli develop to full size at particular loci on one or on several chromosomes. Clearly such contradictory statements cannot be considered complementary evidence that the nucleoli arise from the chromosomes.

Moreover, the relationship of the oocyte nucleoli to the more typical nucleoli of somatic tissues has failed to receive sufficient attention. It is well known that the degree of ploidy in Amphibian somatic tissue can be determined by simply counting the number of nucleoli in resting nuclei (Fankhauser & Humphrey, 1943). This fact follows from the organization of the nucleolus in telophase at a particular locus on one chromosome of the set (Dearing, 1934). Thus from a morphological point of view the oocyte nucleoli pose two distinct problems: (*a*) Where are they formed? (*b*) What relationship, if any, do they bear to the typical somatic nucleolus?



Text-fig. 2. The nucleolus organizing region from three different nuclei of *Amblystoma tigrinum*. In each case both arms of the bivalent are shown; below these are extra-chromosomal nucleoli from the same nuclei.

The available evidence favours the view that the oocyte nucleoli are formed at a single locus on one chromosome by successive growth and detachment. Furthermore, the single locus responsible for the formation of the oocyte nucleoli is the same one which organizes the somatic nucleolus. The evidence for these two statements can be summarized as follows (Gall, 1954*a*):

(*a*) In the two salamanders, *Triturus viridescens* and *Amblystoma tigrinum*, a single locus on one lampbrush chromosome bears objects similar to the extra-chromosomal nucleoli.

(*b*) Though the morphology of the extra-chromosomal nucleoli varies considerably from one preparation to the next, parallel variations are noted in the structures attached to the chromosome (Text-fig. 2).

(*c*) The nucleoli attached to the lampbrush chromosome vary in size from just visible to about 10μ diameter; occasionally they are missing

entirely. Even when it does not bear any nucleoli, the nucleolar chromosome is recognizable by its relative length in the chromosome complement, by its kinetochore position, and by other points of fine structure.

(*d*) The number of extra-chromosomal nucleoli continuously increases with growth of the oocyte.

Statements (*a*) through (*d*) are interpreted to indicate successive growth and detachment of the nucleoli at this one organizing locus.

(*e*) In *Triturus viridescens* a secondary constriction occurs on the mitotic chromosome which corresponds to the lampbrush nucleolar chromosome. Unfortunately, the locus of the somatic nucleolus has never been described for this animal.

(*f*) In *Amblystoma tigrinum* the somatic nucleolus and its corresponding secondary constriction occur at the same locus as the attached nucleoli in the oocyte.

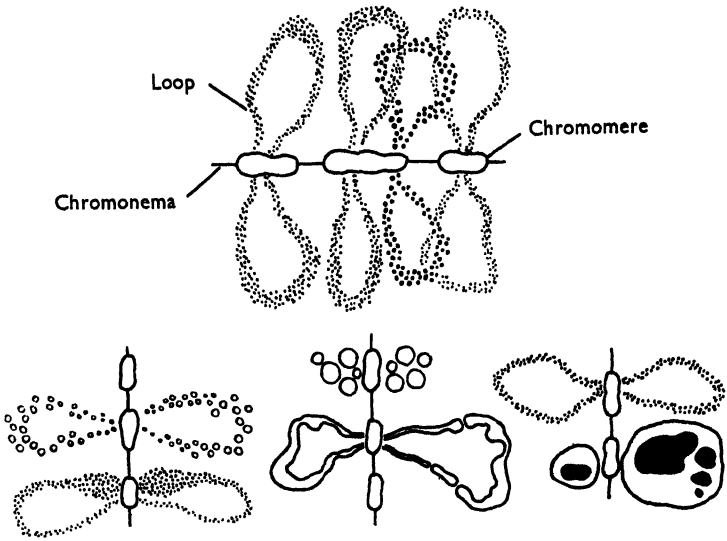
Thus it seems reasonable to believe that the many hundreds of extra-chromosomal oocyte nucleoli are homologous to the chromosomally associated somatic nucleolus in the sense that their production is controlled by the same locus. Demonstration of this truly remarkable condition adds considerable interest to the study of oocyte nucleoli and their relationship to nuclear function.

CHROMOSOMES

Since the earliest observations of Flemming (1882) and Rückert (1892) numerous authors have speculated on the structure of the curious lampbrush chromosomes of vertebrate oocytes. The isolation procedure of Duryee (1937), used more recently in conjunction with the phase-contrast microscope, has now permitted working out of the main structural features of the chromosomes in several species of salamanders. Isolated lampbrush chromosomes surpass the giant salivary-gland chromosomes of Diptera both in size and wealth of detail, and furthermore can be studied quite easily without fixation or staining. Each lampbrush chromosome is actually a pair of homologous strands in a modified diplotene stage of meiosis. The strands are held together at two or more 'contact points' whose relationship to the classical chiasmata of diplotene is somewhat problematical (Gall, 1954*c*). Each individual strand has an axis of Feulgen-positive chromomeres (*c.* 1 μ diameter by 1–5 μ length) joined by an exceedingly fine chromonema. Projecting laterally from the chromomeres are many hundreds of paired loops composed of Feulgen-negative, RNA-containing granules (Text-fig. 3). The loops are responsible for the generally fuzzy appearance of the chromosomes under low magnification which originally gave rise to the designation 'lampbrush'. During growth of the oocytes the loops become

larger, with maximal proliferation in medium-size oocytes (*c.* 0·6–1·2 mm. diameter in *Triturus viridescens*). Still later they decrease in size, disappearing completely as the chromosomes contract in preparation for the first meiotic metaphase.

Most investigators have favoured one of two divergent theories of lampbrush chromosome structure: (*a*) a 'chromonema' hypothesis first proposed by Rückert (1892) and later modified by Ris (1945), according to which the whole chromosome consists of a bundle of loosely associated chromonemata, the loops representing gyres of the individual strands and



Text-fig. 3. Above: diagram of a short chromosome segment to show paired granular loops attached laterally to the central chromomeres. Below: variations in loop structure. The nucleoli (right) and granule clusters (centre) are considered extremes of the general plan of lateral synthesis.

the chromomeres being optical illusions resulting from central overlap of the threads; (*b*) a 'chromomere' hypothesis, stressed by Duryee (1950), and supported by the work of Guyénot & Danon (1953) and Gall (1954*a*), holding that the chromosome has an axis of chromomeres morphologically and chemically distinct from the loops. Neither of these theories, in its simplest form, gives an adequate explanation of lampbrush structure. The 'chromonema' hypothesis, as presented, fails to account for the clear differences between the loops and the central axis in regard to morphology, staining capacity, and solubility. On the other hand, the 'chromomere' hypothesis provides no good explanation of the looped form of the lateral projections. More recent data, particularly from Prof. Callan's laboratory, point the way to a reconciliation of these views. Callan (1955) has suggested

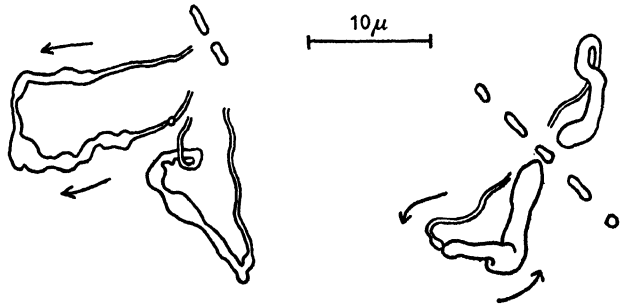
that the loops may have an axis continuous with the exceedingly fine thread seen between successive chromomeres (i.e. the 'chromonema' as I have used the term here; by 'chromonema' Ris (1945) has meant the entire loop, whose diameter varies at different loci from less than one to more than five micra in extreme cases). Callan (1955) has confirmed Duryee's (1950) observation that stretching of lampbrush chromosomes at first separates chromomeres without affecting the loops. However, when a chromosome finally breaks, it does so, not between chromomeres, as one would perhaps expect, but transversely across a chromomere at the base of a pair of loops. At a break, therefore, the apparent axis of the chromosome is interrupted, but the gap is traversed by a pair of extended loops. The phenomenon has been observed repeatedly in chromosomes stretched by microneedles and in those broken accidentally during isolation from the nucleus. A diagrammatic case was shown in my recent paper (Gall, 1954*a*, fig. 21), although its significance was not recognized at the time. The simplest interpretation would seem to be that the submicroscopic strand between chromomeres is actually double, each half continuing through the chromomere and forming an axis to one member of the pair of loops. The bulk of the loop must be some sort of chromosome product accumulated about this axis, as will be discussed later.

The diameter of the chromonema, if indeed it has a uniform diameter, must be only a few hundred Å. units; Tomlin & Callan's (1951) figure of 200 Å. for the diameter of the (double?) strand between chromomeres can be taken as a lower estimate, since this value was found in stretched chromosomes. The postulated axis of the loop remains to be demonstrated by the electron microscope, though it may possibly be visible in some of the pictures of Guyénot & Danon (1953, fig. 13). Ris (1954) describes the loop as composed of a bundle of submicroscopic fibrils, each approximately 500 Å. in diameter, but this work has not yet appeared in full.

It has been known for some time that loop regression in later stages of oogenesis is accompanied by a general increase in chromomere size. Callan (1955) has suggested that during loop regression the postulated loop axis may be complexly folded to form the chromomere. If such is the case, the converse should also be true, i.e. maximal loop formation at a particular locus should correspond to minimal chromomere size. This latter conclusion remains to be verified. The proposed formation of a fine fibril from the chromomere during loop growth finds an analogy in the development of the so-called Balbiani rings of dipteran polytene chromosomes. As Beerman (1952) and Mechelke (1953) have shown in detail, Balbiani rings are formed by a loosening of the structure of particular cross bands, which is to be interpreted as the spinning-out of submicroscopic fibrils. Apparently

in both the lampbrush and polytene chromosomes individual chromomeres can unravel to a tremendous length. In the case of the lampbrush chromosomes, the unravelled threads become coated with a large amount of RNA-protein, making them visible in the light microscope as loops.

A curious feature of loop structure perhaps explicable in terms of a spinning-out of the chromonemal thread is illustrated in Text-fig. 4. Some loops are widest in the segment farthest removed from the chromomere ('type 1') while others are asymmetric, one arm being considerably wider than the other ('type 2', which seems to be the more common). We may



Text-fig. 4. Left: an accurate drawing of a pair of loops of 'type 1' (chromosome 7 of *Triturus viridescens*). Right: a drawing of the upper pair of loops in Pl. 1 C. These are of 'type 2'. In each case the arrows point toward the older parts of the loop.

imagine that chromosome products accumulate about the chromonemal strand as it spins out of the chromomere. In 'type 1', extension of the loop has occurred in both arms, whereas in 'type 2' only one arm of the loop has been involved. In other words, the thicker part of a loop may represent the first formed region.

LOOP FUNCTION

Possible functions of the loops in the synthetic activity of the nucleus have been repeatedly discussed. Most suggestions have actually been somewhat modernized statements of the binuclearity hypothesis of Goldschmidt (1904). Thus the central Feulgen-positive axis of the chromosomes has been considered the genetically important 'idiochromatin', while the loops, whose products are incorporated into the cytoplasm, are viewed as the metabolically active 'trophochromatin'. Dodson (1948) suggests that the lampbrush loops are agents for the synthesis of RNA and enzymes, or other essential developmental substances. Painter & Taylor (1942) suppose that the chromosomal products, containing nucleic acid, are stored in the cytoplasm for rapid synthesis of new chromosomes during cleavage. Duryee (1950)

goes even further and imagines that each loop consists of some specific compound needed during later development. Before any such statements can be evaluated critically we must know considerably more about the chemical and physical organization of the loops.

As already mentioned each loop is composed of a great many small Feulgen-negative granules which contain cytochemically demonstrable quantities of RNA (earlier staining experiments now supported by unpublished U.V. absorption data of Swift and Gall). The size of the granules as well as their brilliance under phase contrast varies from locus to locus. Most loops consist of numerous very fine granules of low optical contrast, but others exhibit a smaller number of large, brilliant spheres or irregular masses of material (Text-fig. 3). Likewise the loops exhibit considerable variation in size. These differences cannot be explained as different stages in the formation of a single type of loop, since they are characteristic of particular chromosomal loci. In fact the chromosomes can be mapped on the basis of loop structure alone, and particularly striking loops are used regularly in chromosome identification (in addition, of course, to relative chromosome lengths, kinetochore position, and chromomere pattern). Although the most conspicuous loops may be composed of granules a micron or more in diameter, relatively few indeed possess such a structure. Loop granules in general verge on the limit of resolution; many loops which appear hazy without distinct speckling must be made of granules less than $0{\cdot}2\,\mu$ in diameter. Unfortunately, the few electron micrographs now available are not sufficiently clear for a determination of the fine structure of the loops (Pl. 1 A).

On first consideration it might seem that each chromosome locus produces material which is simply stored as a pair of loops until late in oocyte development. The dispersal of the loops before metaphase I would then permit the constituent granules to become part of the nucleoplasm and so be incorporated into the general cytoplasm after nuclear membrane breakdown. However, a more dynamic interpretation of loop formation is possible, involving continuous dispersal of granules into the nucleoplasm during oocyte development. On such a view, growth of the loops would occur when the rate of granule production exceeded that of dispersal, while the final disappearance of the loops would represent the opposite condition. This dynamic interpretation is suggested by the structure of the nucleoplasm itself, by analogy with the nucleoli, and by direct observation of granule dispersal.

As already shown the nucleoplasm represents 90% or more of the total nuclear volume in middle size or full-grown oocytes. It seems most unlikely to me that all this material should be simply an inert bathing medium

for the chromosomes. That it represents, on the other hand, an accumulation of free loop granules is suggested both by its structure and its chemical composition. In addition to the 1000 or more nucleoli described above the nucleoplasm contains literally hundreds of thousands of minute granules ranging from the barely visible to 4 or 5 μ in diameter. All of these granules, as well as the background nucleoplasm (submicroscopically granular?), when fixed and stained exhibit positive tests for RNA. The nucleoplasm, as might be expected, contains in addition a great deal of protein, since an amino-acid analysis can be made by paper chromatography using only 30–40 nuclei (Brown, Callan & Leaf, 1950; personal observation). Since the granules found in the nucleoplasm can be matched by granules of similar morphology and chemical composition on the chromosomes, it is not difficult to suppose that they are equivalent structures.

Such a formulation simply extends to the loop granules the same property of successive growth and detachment postulated for the nucleoli. That the nucleoli may not be so fundamentally different from the loops in their mode of formation is suggested also by the production at many loci of structures which, in a sense, seem to be intermediate between typical loops and typical nucleoli. Reference to Text-fig. 3 will indicate better than lengthy descriptions what is meant. The chromosomes bear not only the customary finely granular loops, but also solid bands of material, large granules in the form of loops, clusters of granules with no evident indication of loop form (although often demonstrably paired like the loops), and many other variations. In this light the nucleoli seem to be only the extreme in a range of structures, especially since they are also paired at times and, like the loops, are definitely situated lateral to the main chromosomal axis.

Direct evidence for continuous loop breakdown is available from observations on some of the curious solid loops like those shown in Text-figs. 3 and 4 and Pl. 1 C. These loops are occasionally visible within the intact nucleus, although the chromosomes are generally quite difficult to study without isolation. In some instances a cloud of large granules has been noted surrounding the loops and extending out as much as 100 μ on all sides. The granules are of the same optical contrast as the loops and must represent loop fragments slowly dispersing into the gelatinous nucleoplasm. Callan (1952) described a radial distribution of granules within the nucleus, which may well be another expression of this same phenomenon.

As a general statement we may say that the chromosomes synthesize products more or less continuously during oocyte development. Ordinarily these products are organized into paired loops consisting of locus-specific RNA-protein granules. However, the granules are constantly breaking away from the loops to enter the nucleoplasm, which contains therefore a

complex aggregate of particles theoretically divisible into qualitatively differing classes. The various types of loops, clusters of granules, nucleoli, etc., found attached to the chromosomes represent merely different forms assumed by the chromosome products (cf. Gates, 1942; Duryee, 1950).

DISCUSSION

The morphological features of the amphibian germinal vesicle summarized in the preceding paragraphs have suggested the following scheme of nuclear synthesis. Chromosomal products appear during oogenesis as discrete ribonucleoprotein (RNP) granules, the vast majority of which are on the borderline of visibility in the light microscope, but which range up to a micron or more in diameter. The granules are characteristically organized into pairs of loop-like projections extending laterally from the chromosome axis. The many hundreds of loops arising from a single chromosome differ from one another in terms of relative length, granule size, optical contrast, etc., suggesting a heterogeneity of chemical composition. Furthermore, it is likely that material is being given off from the loops into the nucleoplasm all during oogenesis, and that new loop granules arise continuously.

This system of particulate chromosome products in the oocyte nucleus immediately poses many interesting questions regarding the relationship of the chromosomes to protein synthesis. That proteins accumulate in the loops, nucleoli, and nucleoplasm during oocyte development is certain from solubility, staining, and digestion experiments (Gersh, 1940; Brachet, 1945; Dodson, 1948; Gall, 1954*a*) and from amino-acid analysis of the nuclear contents (Brown *et al.* 1950). The recent experiments of Ficq (1953) on the incorporation of ^{14}C-labelled glycine into the protein and nucleic acids of starfish oocyte nuclei demonstrate a very rapid uptake of isotope into the nucleolus, about 100 times faster than into the cytoplasm. It is also well known that ^{32}P incorporation into the RNA fraction of the nucleus proceeds at a very high rate (Barnum *et al.* 1953), especially in the nucleolus (Taylor, 1953; Stich & Hämmerling, 1953). There are, therefore, strong suggestions that synthesis of ribonucleoproteins takes place in the nucleus. Whether all of this material is formed under the direct control of the chromosomal loci, or whether the products themselves are capable of further synthesis after leaving the chromosomes is an intriguing question for further research.

That the loop materials eventually leave the nucleus cannot be doubted, since observations on a wide variety of forms has shown that the breakdown of the germinal vesicle is followed by a general mixing of the nuclear contents with the cytoplasm. On the other hand, it would be particularly

interesting to know whether the nuclear products can actually pass through the nuclear membrane during oogenesis. Evidence exists that the nuclear membrane is permeable to certain protein molecules (Anderson, 1953), and reports are common in the cytological literature that nucleoli or other visible particles may penetrate the intact membrane (e.g. Schrader & Leuchtenberger, 1952; Duryee, 1950; Chaudhry, 1951; Wittek, 1952). Electron-microscope observations on the germinal vesicle membrane (Callan & Tomlin, 1950; Bovey, 1952; Gall, 1954*b*) have so far shed no light on this exceedingly important problem. The importance of the nuclear contribution to the egg cytoplasm at the breakdown of the germinal vesicle was long ago stressed by cytologists and embryologists alike. Indeed, it was explicitly suggested that the basic staining nuclear 'chromidia' gave rise to the cytoplasmic 'microsomes', although the latter term then encompassed what we now know as microsomes, mitochondria, and various types of secretion granules.

It is tempting to suppose that the nuclear RNP fraction may be associated in the cytoplasm with further protein synthesis. Such a suggestion has been made many times in the past, as for instance in the able discussions of Caspersson (1950) and Brachet (1952*a*, *b*). It is well known that cytoplasmic protein formation is associated with the RNA-rich microsome or small particle fraction (e.g. Hultin, 1950; Borsook *et al.* 1950; Siekevitz, 1952). The origin of this fraction and its organization in the intact cell are only poorly understood, so that any suggestions of a nuclear contribution must be tentative. However, it seems reasonable to suppose that the ultimate genetic control of cytoplasmic syntheses must reside in special chromosomal products; the locus-specific RNP materials of the lampbrush chromosomes could perhaps be among such products.

I should like to express my gratitude to Dr H. Swift and Dr D. Mazia who kindly read the manuscript of this paper and offered valuable criticisms. I feel especially indebted to Prof. H. G. Callan who has generously discussed with me much of his unpublished work on lampbrush chromosomes; without his helpful comments this paper could not have been written.

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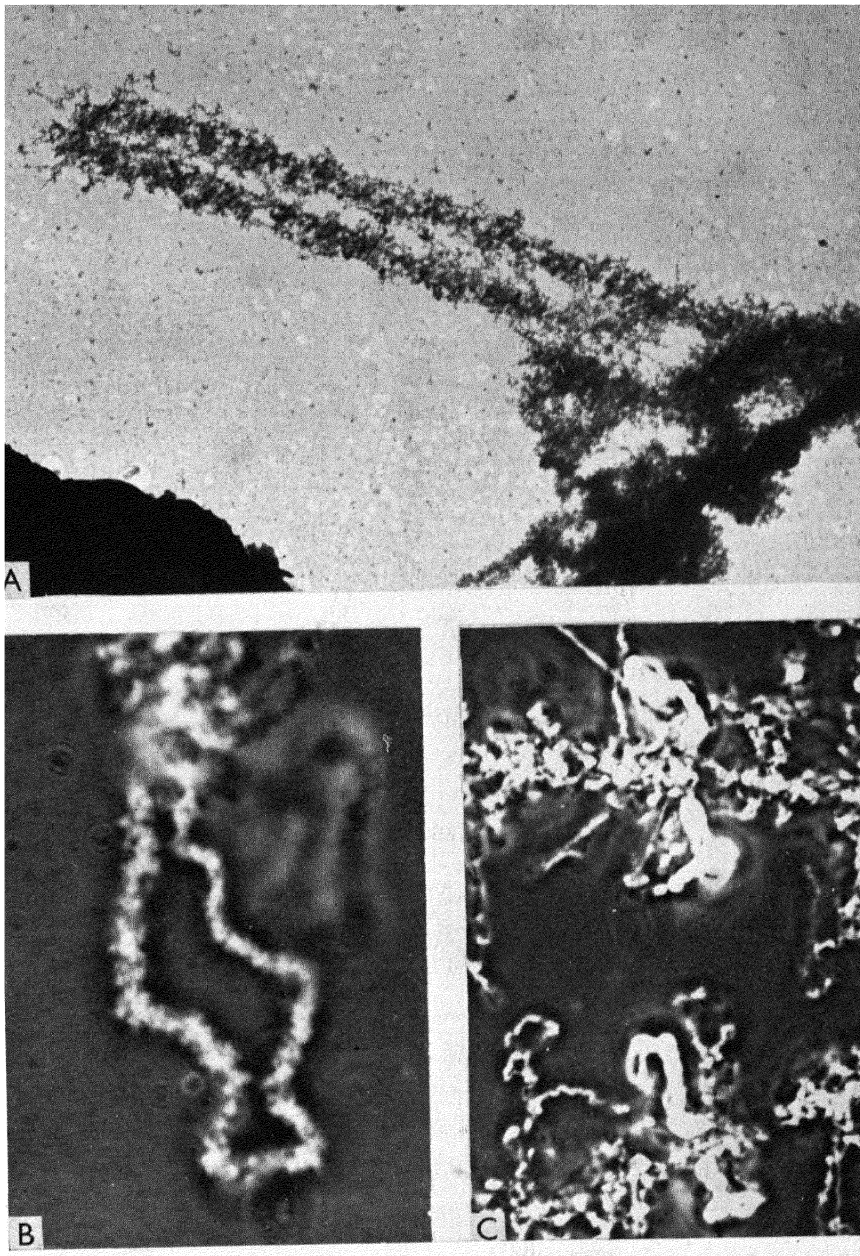
EXPLANATION OF PLATE 1

A. A single lampbrush loop viewed with the electron microscope. Fixed in formaldehyde vapour. Dehydrated according to Anderson's critical point method. Unshadowed. ×6700.

B. A single lampbrush loop viewed with the phase-contrast microscope. Note the finely granular structure. The second loop of this pair is out of focus to the upper right. Fixed in osmic vapour. ×2900.

C. Both arms of a single bivalent to show paired loops at corresponding loci. Compare Text-fig. 4 for a drawing of the upper pair. Fixed in formaldehyde vapour. Phase contrast. ×1460.



For explanation see p. 370

(*Facing p. 370*)