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THE CLASSIFICATION OF FERNS

By R. E. HOLTTUM, *Botanic Gardens, Singapore*

(*Received* 15 *July* 1948)

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I. INTRODUCTION

The simultaneous publication by Copeland of a book on the genera of f
of a paper by myself presenting a critical revision of Christensen'
of classification, offers a suitable opportunity for a discussion of
of our knowledge of the subject. Such a discussion is here atte
literature is fully cited in Christensen (1905–34) and Copeland

Some ten thousand species of ferns have been described
proportion which are regarded as primitive have received
logical study, and these are admirably treated by
quarters of the described species of ferns, nearly all of t
subtropics, all until lately united in a vast family Polyp
much less attention from morphologists. Most of
described in brief taxonomic formulae designed me
their presumed allies. The descriptions are not in
purpose, in a great many cases, and there may
undetected because the species have been inade

These 'modern' ferns are so extremely var
their parts, that no one man could pretend t
even as to their assignation to genera. Indee
of the genera have been reasonably well disti
first full conspectus of a great deal of work (1
ast forty years.

he early attempts at definition of fern
e, structure and arrangement of
ought other factors into conside
t, and it was not until the
and Diels, that a mo
form of sori

Hooker) had developed on several different lines of evolution gave a radically new viewpoint. This necessitated the examination of the structure and development of all sori in much greater detail, to discover small differences which might give evidence of evolutionary origin, and also in the search for other characters which might give indications of the same kind. This search involves examination of all parts of the fern plant, both external and internal. It is a large task, and, as above indicated, has not progressed very far in the great majority of ferns. Bower (1923) has given a survey of the ground to be covered. Only when more ferns have been examined on this intensive scale shall we have a satisfactory basis for our classification. Probably some factors not mentioned by Bower may also be important in certain groups of ferns; certainly different sets of factors will be of greatest importance in different groups. In interpreting the results we shall also have to remember that not only in sori, but also in other parts of the plant, similar forms have probably developed on different lines of evolution. Similarity of environment may have favoured similar adaptations in widely different ferns. It is essential to know some-
of ecology if we are to understand our problem fully.

re divided into eusporangiate and leptosporangiate. The former comprise
inct groups, Ophioglossales and Marattiales, neither of them very
o the leptosporangiate ferns. I do not attempt to discuss the
is in the present article.

CHARACTERS OF A PRIMITIVE FERN

nerally adopted hypothesis that leptosporangiate ferns have
t will help in our understanding of the ferns of to-day
tral form was probably like, and in what kinds of ways
arted from that form. The developments have mainly
fferent kinds of ferns. Some may retain a primitive
ced in vascular anatomy or soral condition or some
reverse may be true. Some are primitive in most of
d to be nearest the ancestral form. Some are
spects, and these must be highly evolved. But
advanced characters, and we have no means of
e guess how many forms have been developed
ferns we have to-day. Probably all that have
m their primitive ancestors.
ves (megaphylls) were derived from branch-
ing specialized to photosynthetic and repr
e plant becoming specialized as rhizom
re would thus have been originall
lk (stipe), but as rhizome and le
lly develop. I suggest beginn
which was already di

The following is a summary of the characters of such a hypothetical primitive fern, based on a study of living ferns:

Rhizome rather slender, creeping, dichotomously branching, bearing alternate 2-ranked leaves on its upper surface and roots on the lower surface; vascular system a protostele; surface covered with hairs.

Fronds large, amply dichotomously branched, the stipe containing a single vascular strand, the ultimate leaflets narrow, each containing a single vein, surfaces more or less hairy, at least when young.

Sori terminal on some of the leaflets (i.e. at the end of a vein); *sporangia* rather large, with primitive annulus, few in each sorus, opening simultaneously.

III. EVOLUTIONARY DEVELOPMENTS FROM THE PRIMITIVE FORM

Rhizome. This may become thicker and shorter, with the leaves closer together; it may develop special ways of branching, the branches associated with leaf-bases; it may remain dorsiventral in structure but have more than two ranks of leaves on its upper surface, or it may become radial in structure and more or less ascending, the final stage being an erect stock or trunk. Vascular anatomy will develop according to external form. Bower (1923) has summarized the stages of development of solenostele (hollow cylinder) and dictyostele, but he has not emphasized the significance of dorsiventral structure, which seems to me important; nor have morphologists done justice to the kind of development seen in *Davallia*, where the rhizome remains long-creeping and dorsiventral, but the solenostele becomes dissected into a kind of lattice instead of a continuous hollow cylinder (Bower, 1928, p. 17). It is misleading to call this a dictyostele, as is often done. The development of sclerotic tissue, giving support in various ways, may often occur; this has been insufficiently studied and may give useful indications of relationships, but it needs to be interpreted with care, and with some knowledge of the environmental conditions under which the plant grows.

Hairs on the rhizome may give place to scales. The transition can be seen in various groups of ferns, and has certainly developed on several different evolutionary lines. The transition may come by thickening and flattening of an unbranched hair, or by the development of peltate scales from stellate hairs. The form of the base of the scale, the shape and formation of marginal teeth, the precise characters of marginal and superficial hairs, the nature of thickening of the cell-walls of the scale, are all possible indicators of affinity. Much more detailed study needs to be made of these characters in the majority of ferns.

Stipe (petiole). This may become jointed at the base, allowing of the complete shedding of the frond in (unfavourable) resting seasons; this has occurred in several quite distinct groups of ferns. The position and structure of the joint (nature of absciss-layer) may be significant; anatomy of such joints has been little if at all investigated. The vascular anatomy of the stipe is significant. The single strand of a primitive fern usually gives place to two or more; the number of strands at the

base of the stipe and their origin from the rhizome stele, also the branching or fusion of the strands in the stipe need to be accurately observed.

Aerating tissue in the stipe needs much more investigation. There is usually a line down each side of the petiole along which thin-walled tissue reaches the surface; this may be a primitive character. The line is sometimes not continuous, and along it may be prominent aerophores (often but not always in conjunction with reduced leaflets) or glands, which have different function and structure in different groups of ferns; they are functional only in very young fronds.

Frond form. It is evident that at an early stage of evolution the dichotomous branching of the frond gave place to pinnate branching; the transition, by way of alternate unequal dichotomies, is a simple one. Most ferns retain dichotomous branching of some of the smaller veins, but only in the groups of *Schizaea*, *Matonia* and *Dipteris* and in the peculiar genus *Rhipidopteris* has dichotomy of the main branching persisted, the smaller veins being more or less pinnate.

The lamina of the fern frond, originally supposed to have had a single vascular bundle (vein) in each lobe, as still seen in Hymenophyllaceae, developed by fusion of the lobes, giving rise to leaflets each with several veins. This fusion process has occurred to some extent in most groups of ferns, in some more than others. The ultimate stage of this development is a simple unlobed frond, and such fronds have certainly been evolved on several quite distinct evolutionary lines. Along with the increase in webbing of the lamina has gone the development in most cases of a more or less reticulate venation. In a few cases the stage of simple frond has been reached with little or no anastomosis of veins; *Oleandra* has quite free veins, and there is only slight anastomosis in *Elaphoglossum* and *Syngramma*, all three representing (in my view) different evolutionary lines. The number of possible forms of anastomosis is not large, and closely similar complex forms have developed independently in *Tectaria* (*Aspidium*) and in the polypodioid genera. In a few cases reduction of the simple lamina has probably proceeded so far that the anastomosing venation has again to give rise to a free venation; an example is *Monogramma*.

A peculiar condition is seen in the polypodioid ferns, in which it seems that deeply lobed and even simply pinnate forms have developed from simple fronds which resulted from a simplification of the *Dipteris* type.

The general shape and mode of branching of fronds has developed in several ways. In some the general shape is narrow, with lateral branches (pinnae) more or less reduced (sometimes much reduced) towards the base of the frond; in others the general shape is deltoid, with greatest development of the basal pair of pinnae, often also of the leaflets along the basiscopic side of those pinnae. The shape of ultimate leaflets, whether symmetrical or not at the base, and the shape of their lobes, and the arrangement of lobes or teeth on their edges have also developed in various ways. Sporeling plants may have leaves which give some indication of an ancestral form, especially of ferns which when adult have simple fronds. The bathyphylls of *Teratophyllum*, and the bipinnate fronds of cultivated *Nephrolepis*, may give similar indications.

The structure of the rachises, their way of branching, and the structure of attachment of leaflets to the rachises bearing them, appear to me important as indicating evolutionary lines. These characters are only shown in a well-developed state by much-branched fronds, but I think they are significant, and that one particular structure is important as a factor indicating relationship between the *Dennstaedtia* and *Dryopteris* groups of genera.

Scales on fronds have developed in much the same ways as on rhizomes, but are smaller, and sometimes peculiar forms may occur (as the bullate scales on leaflets of *Cyathea*, and the peltate scales on some polypodioid genera). Hairs are often present as well as scales, and particular kinds of hairs are characteristic of certain groups of ferns (e.g. in *Ctenitis*, for long united to *Dryopteris* and other genera).

Sori. Bower (1923, ch. XII) has shown that the primitive ferns all had sori of few sporangia developing simultaneously, and that on more than one line has come a transition to a 'mixed' condition with many sporangia not developing simultaneously; a 'gradate' intermediate condition may also occur. The vast majority of ferns have mixed sori.

Though we may postulate a marginal primitive position (at the ends of veins) for fern sori, the transition to a position on the lower surface of the frond occurred very early in some lines of evolution, and we have no indication of how the change occurred; such ferns are Gleicheniaceae and Polypodiaceae (in the restricted sense). Bower has called these ferns Superficiales.

In other ferns the primitive soral form appears to have had a protective flap on both sides, the two flaps sometimes partly joined to form a cup. It has been shown by Bower (1928, p. 18) how the superficial sori of *Davallia* and other genera can have been evolved from such marginal sori protected by two flaps; the one flap becomes thicker and in effect forms an extension of the leaf-margin beyond the end of the vein which bears the sorus, and the other flap remains thin, being then called an indusium. This indusium may be attached at the base only, or along the sides (thus forming a pouch); if attached at the base, it may develop a broad or deeply cordate form, and many forms may be found among ferns which are obviously nearly allied, especially in the genera allied to *Davallia*. Alternately, the indusium (lower flap) may be aborted, and the new edge of the frond formed by the upper flap may be more or less reflexed to protect the young sorus.

After this transition from a marginal to a superficial position had been achieved, various other developments were possible. By more than one series of changes the sorus may have become dorsal instead of terminal on its vein, or may have become elongate along its vein (e.g. it seems likely that two quite different evolutionary processes gave rise to the elongate indusiate sori of *Asplenium* and *Athyrium*). When webbing of the lamina gives rise to anastomosis of veins, sori may remain terminal on free veins or be seated upon anastomosing veins, or sometimes at vein-junctions. At any stage of evolution the indusium may be entirely lost. When this has occurred, there has been a tendency for sori to spread along the veins. This has been especially noteworthy in Polypodiaceae (*sensu stricto*), in which naked sori

have probably existed for a longer time than in other groups of ferns; but it occurs also, for example, in *Tectaria* (*Aspidium*) and allied genera, in species which have lost indusia. It has occurred also in the gymnogrammoid ferns, such as *Coniogramme* and *Syngramma*. The ultimate condition of spreading of sori is throughout the length of all veins; then, if the lamina of the fertile fronds is reduced in area, we have the acrostichoid condition (first described in *Acrostichum*). Sometimes the sporangia may also spread over the surface of the lamina between the veins. The acrostichoid condition has developed on many different lines of evolution. Some ferns which have acrostichoid fertile fronds are otherwise extremely like other ferns which have broader fertile fronds with well-developed indusia. For example, the genera *Stenosemia* and *Quercifilix* are vegetatively indistinguishable from *Tectaria*, but they appear to be most nearly related to different species of *Tectaria*. Some ferns have fertile fronds much reduced in width, but the sori still just separate.

Another kind of evolutionary soral change is the production of fusion-sori, especially of submarginal fusion-sori, as in *Pteridium*. This is presumed to have occurred by lateral spreading of each sorus, terminal on its vein, so that the united sori produce one continuous sorus just within the margin, the indusia of the sori also joining to form one continuous flap. All stages of this development can be seen within the genus *Lindsaya*. The same development also occurred in the pteroid ferns and in *Nephrolepis*. A marginal sorus occurs in some gymnogrammoid ferns, and some of them are very like *Pteris* in aspect; whether these represent two different developments is a debatable matter. The *Blechnum* fusion-sorus is not marginal, and probably had a different origin.

In the more highly developed sori, especially in fusion-sori and in the acrostichoid state, special additional vascular tissue has been evolved to provide for the needs of the sori. The details of development of such tissue may vary considerably among species of near alliance, depending on the width of lamina which has to be served; but it is found that in some acrostichoid ferns such tissue is completely lacking.

Sporangia. Bower (1923, ch. XIII) shows how all leptosporangiate sporangia can be regarded as evolved from the types found in Gleicheniaceae and Schizaeaceae, and that the oblique annulus is a primitive condition. The vertical annulus, interrupted (or partially interrupted) at the stalk, is found in the great majority of ferns, and again has probably evolved on several lines. Precise details of sporangial structure are known for a small proportion of ferns only. Sporangia sometimes bear hairs (sometimes glandular hairs) of the same form as those on the surface of the fronds which bear them; notably in the exindusiate sori of the *Grammitis* and *Thelypteris* alliances. Paraphyses sometimes occur among sporangia, especially in naked sori; sometimes they are like hairs elsewhere on the frond, and sometimes they are perhaps abortive sporangia. Umbrella-shaped paraphyses in some of the polypodioid ferns are clearly to be derived from stellate hairs which occur in some such ferns.

Spores. There are two principal spore shapes, bilateral and tetrahedral, caused by the way in which successive divisions of the spore-mother-cell take place. Probably

both forms have existed from a very early stage. In most groups of ferns existing to-day, either one form or the other is found; and if a single species in a genus has different spore shape from all the rest, it is to be regarded with suspicion. It seems that, apart from *Loxogramme*, the polypodioid ferns can be sharply distinguished from *Grammitis* and its allies on spore form. But in other groups it appears that both spore forms may occur. The presence of a convoluted perispore is a distinctive character of certain groups of ferns, lacking in others. It seems probable that this is not a primitive character, and possible that it has developed on more than one evolutionary line. Within some genera which bear it, the perispore varies much in form, and the convoluted wing may be broken up into separate small wings or these reduced to spines (all conditions are found in such different genera as *Cyclosorus* and *Lomariopsis*). Many more spore studies need to be made, and a more detailed examination of the structure and development of perispores.

Prothalli. Little has yet been published on the comparative morphology of prothalli of the majority of ferns. There must at least be a considerable degree of physiological specialization of prothalli to different habitats. In such simple organisms, such specialization may overshadow distinctive features which might indicate evolutionary lines.

IV. THE ESTABLISHMENT OF GENERA AND GROUPS OF HIGHER RANK

Having regard to all these evolutionary possibilities, we have to consider the problem of recognizing natural genera and of arranging these in natural groups of higher rank. The problem of genera is not difficult, once we have established the principle that soral form is of no more significance than any other character, and that species having soral differences may yet be closely allied. The old sorus-genera were divided by Hooker into sections which were based on vegetative characters. These sections are in many cases good natural genera, or in some cases subgenera of two different sorus-genera may be united (e.g. when they differ only in presence or absence of an indusium). In this way, a large number of natural genera are not difficult to recognize, each presenting a fairly uniform set of vegetative characters, combined (in some cases) with small soral differences. But the complex groups of genera included under the names *Dryopteris* and *Polypodium* by Christensen (1905–34) are not easy to disentangle, and need very careful analysis. Christensen himself (1912, 1919) showed how the *Dryopteris* group might be subdivided by consideration mainly of scales and hairs and frond architecture, though he never made a full formal subdivision of the group. The resulting genera, once separated, are seen to be natural; and though not all of them have yet been clearly distinguished, the main divisions appear to have been satisfactorily established. The genera of the *Polypodium* group have been similarly disentangled, mainly by Copeland and Ching.

Having now distinguished our genera, the problem arises of combining them into allied groups of higher rank. This is a much more difficult process, and one upon which there is yet no general agreement. The principal recent schemes are those of

Bower (1928), Christensen (1938), Ching (1940), Copeland (1947) and myself (Holttum, 1947). The extent to which these schemes differ is a measure of the lack of precise information upon which to found such schemes; differences are due partly also to differences of emphasis on different kinds of characters, and partly I think to the difficulty for one mind to hold in impartial review such a vast assemblage of data. I am naturally prejudiced in favour of my own scheme, but would emphasize that it is purely tentative, put forth as a basis for further work; it is bound to include many mistakes and some bad guesses.

If I may presume to pass judgement on the other schemes, I would suggest the following. Bower's scheme is unsatisfactory because he had not examined enough different species in detail, and took other people's unsatisfactory genera too much on trust. Christensen's scheme is a great improvement on that of Bower; but he seems to have accepted uncritically certain ideas of Bower's, notably the affinity of the dryopteroid genera to Cyatheaceae. Christensen had a vastly more extensive knowledge of fern species than Bower, and his treatment of the dryopteroid and polypodioid genera is especially important. The scheme is no more than sketched out; it contains no detailed discussions or descriptions of genera.

Both Bower and Christensen agree with earlier authors in grouping all ferns except the obviously more primitive genera (those in Bower's second volume) as one family Polypodiaceae. Ching's scheme proposes thirty-three families, but these appear to me very unequal in character and too numerous to display well the evolutionary lines within the assemblage of modern ferns. I have not been able to study the scheme in detail; very few copies exist. Copeland's scheme divides the Polypodiaceae of Bower and Christensen into eight families; my own scheme divides it into five. Copeland's main families are Pteridaceae and Aspidiaceae; I consider both to contain a mixture of not nearly allied genera. In many cases Copeland gives no argument for placing his genera, and he appears to me to have omitted consideration of a number of points discussed in my own paper (published almost simultaneously with Copeland's book). Copeland's is the only modern work in which all the genera of ferns are described and the type species of each cited, and is of great value in this respect. The great majority of the genera will stand, though a few seem to me superfluous, and there is room for argument about the selection of a few of the generic names.

V. COMPARISON OF RECENT SCHEMES OF CLASSIFICATION

Taking the ferns dealt with in Bower (1928), these are divided by him into two main groups, Superficiales and Marginales. Under Superficiales, probably derived from Gleicheniaceae, Bower places *Dryopteris*, *Tectaria* and allies (including *Thelypteris*); *Asplenium* and *Athyrium*; *Blechnum* and allies, including the ferns confused as *Stenochlaena*. He places in a distinct group of Superficiales the dipteroid ferns and some polypodioids.

Under Marginales, derived from a type something like *Dennstaedtia*, Bower places *Hypolepis*, the davallioid and pteroid ferns. In addition to these, he distin-

guishes the gymnogrammoid ferns, derived from some primitive groups related to Schizaeaceae and Osmundaceae, but showing some (convergent) resemblances to the pteroid ferns.

Christensen's scheme (1938) follows Bower's in the main. He divides Polypodiaceae into fifteen subfamilies. He retains *Asplenium* and *Athyrium* in one subfamily, but distinguishes the two clearly (as Bower did not). He gives a full analysis of the dryopteroid ferns and shows the distinctness of *Thelypteris* and its near allies (which Bower does not clearly mention). He rejects Bower's derivation of *Phyllitis* from a blechnoid type, and removes 'Stenochlaena' from the blechnoid

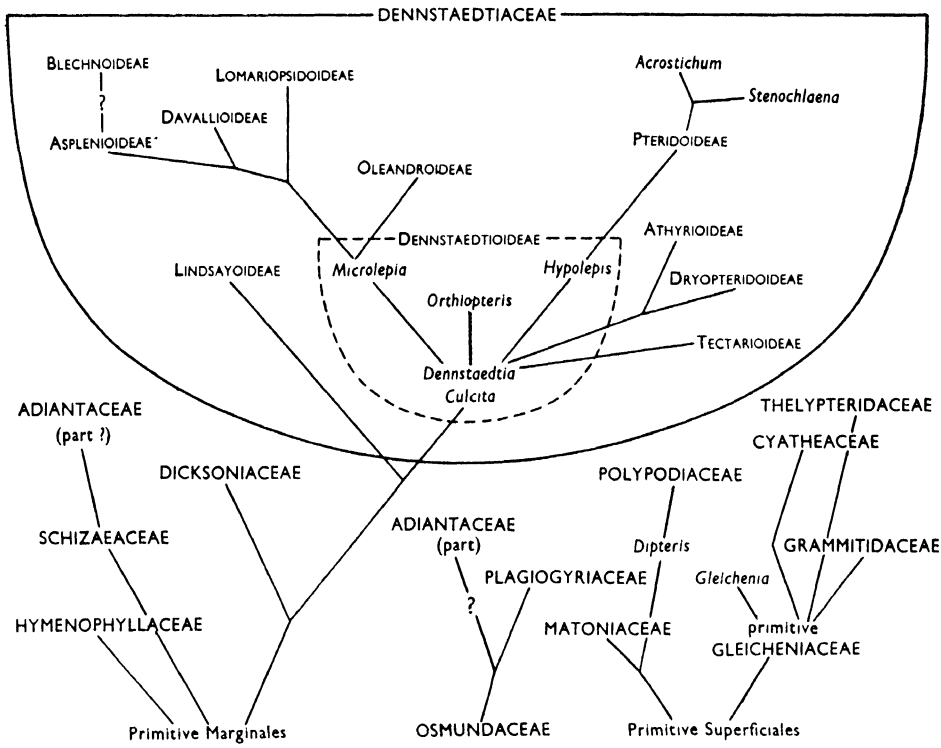

Fig. 1. Diagram showing the interrelations of the various groups of ferns according to the scheme of classification outlined in the present article.

group, dividing it (according to my earlier work) into *Stenochlaena* proper (associated with *Acrostichum*) and *Lomariopsis* and *Teratophyllum* (associated with *Bolbitis*, etc., as acrostichoid genera of probably dryopteroid origin). He maintains the position given by Bower to the gymnogrammoid ferns, with minor alterations in their subgrouping. He gives a full analysis for the first time of the polypodioid ferns, pointing out the peculiarity of the *Grammitis* group; and places *Elaphoglossum* in a subfamily of its own at the end.

My own scheme unites all Bower's Superficiales, except *Thelypteris* (and its allies) and the *Dipteris-Polypodium* ferns, with the Marginales, in a single large family Dennstaedtiaceae, with eleven subfamilies; I believe all to have originated

from *Dennstaedtia*-like ancestors with marginal sori, and discuss reasons for disagreeing with Bower's suggestion that the primitive dryopteroid genera show affinity with Cyatheaceae. I separate *Asplenium* and *Athyrium* into distinct subfamilies, considering the former to be more nearly related to *Davallia* and the latter to *Dryopteris*; I interpret Bower's *Blechnum-Asplenium* series in the reverse direction; and place the acrostichoid genera *Lomariopsis*, *Teratophyllum*, etc., in a separate subfamily, believed to be allied to *Davallia*, and adds to them *Elaphoglossum*.

My scheme restricts the family Polypodiaceae to the strictly polypodioid genera and *Dipteris*, separating the *Grammitis* group as another family. It places *Thelypteris* and near allies in a family Thelypteridaceae, suggesting that they may be allied to Gleicheniaceae and Cyatheaceae, though the true dryopteroid ferns (with which *Thelypteris* is usually united) are not. It leaves the gymnogrammoid ferns as treated by Christensen and Bower, only adding *Taenitis* to them and uniting them with Vittarioideae in a family Adiantaceae. It leaves the Onocleoideae unplaced, suggesting that they are not nearly allied either to Cyatheaceae or Dennstaedtiaceae.

Excluding again the more primitive families, about which there is general agreement, Copeland recognizes the following: Pteridaceae, Parkeriaceae, Hymenophyllopsidaceae, Davalliaceae, Aspidiaceae, Blechnaceae, Aspleniaceae, Polypodiaceae and Vittariaceae.

Under Pteridaceae he has the ferns usually separated as Dicksoniaceae, also the *Dennstaedtia*, *Lindsaya* and *Pteris* groups (placed by me as subfamilies of Dennstaedtiaceae), and all the Gymnogrammoid ferns. Parkeriaceae is maintained for the single genus *Ceratopteris* (placed as a gymnogrammoid by Bower). Hymenophyllopsidaceae includes a single small genus, also separated by Christensen.

Davalliaceae comprises *Davallia* and its near allies, also *Oleandra*, *Nephrolepis* and *Arthropteris*. Copeland objects to a near association of *Davallia* with *Microlepia*. He places *Nephrolepis* as the oldest genus in his Davalliaceae; but I would consider it on the whole much more specialized than *Davallia*.

Aspidiaceae includes (*a*) onocleioid ferns (unplaced in my scheme); (*b*) *Dryopteris* group; (*c*) *Thelypteris* group (the name *Lastrea* is used instead of *Thelypteris*); (*d*) *Lomariopsis* group; (*e*) *Athyrium* group. Copeland believes the primitive members of his Aspidiaceae to have had an origin cognate with Cyatheaceae.

Blechnaceae includes the usual *Blechnum* group and also *Stenochlaena* (*sensu stricto*) which my scheme places with *Acrostichum* in the *Pteris* group. Copeland thinks *Blechnum* related to *Athyrium*.

Aspleniaceae includes the usual asplenioid ferns, excluding *Athyrium*.

Polypodiaceae includes *Dipteris*, the strictly polypodioid ferns, and the *Grammitis* group. Copeland considers that *Loxogramme* should be associated with *Grammitis*.

It will thus be seen that Copeland's scheme offers a number of differences in detail from my own, and some incompatibilities. In the remainder of this article

I have set forth my own scheme, attempting to summarize the features of each group of genera as I see it, without arguments, which are to be found in the paper of 1947. For the sake of completeness, I have included here also brief descriptions of the more primitive families of leptosporangiate ferns.

Copeland discusses at length the probable antarctic origin of most living ferns ('practically the whole fern world of the tropics is descended from the ferns of old Antarctica'). He considers that most pantropic groups owe their present distribution to separate migrations northwards from the antarctic, and 'the most primitive, even archaic, representatives of many groups are to be found as near as is now possible to their antarctic source'. This subject needs more study; but I suggest that the austral representatives of Gleicheniaceae, for example, are not the most primitive. One needs to have some criterion of what is primitive; I have tried to give indications of this in the early part of the present paper. Most ferns show some primitive characters, and the southern members of Gleicheniaceae have some; but on the whole, with Bower (1926), I should consider them specialized xerophytes, and see in some tropical members the most primitive living condition of the family. But of course that does not exclude the possibility that austral representatives are relics showing lines of migration, still existing in the south, because they have become suitably adapted. The matter is a complex one, and I do not feel competent to discuss it further.

VI. THE AUTHOR'S CLASSIFICATION

1. Osmundaceae

Terrestrial ferns with erect radially symmetrical rootstock bearing a close rosette of fronds, not scaly; vascular strand of stipe C-shape in section, base of stipe bearing stipule-like organs; fronds pinnate to bipinnate or more deeply dissected, veins free; sporangia borne on specialized reduced leaflets which have no lamina (*Osmunda*) or closely placed along the veins of ultimate leaflets, large, with rudimentary annulus; spores green (in *Osmunda* at least).

Genera: *Osmunda, Todea, Leptopteris.*

Bower places this on the border-line between eusporangiate and leptosporangiate ferns. It is known to be an ancient family (there are Palaeozoic fossils), and the sporangia are very primitive in form; the frond form of *Leptopteris* is primitive, *Osmunda* being more specialized. The erect rootstock is quite highly organized. The stipule-like organs at the base of each stipe resemble similar organs in eusporangiate ferns. It seems possible that *Plagiogyria* and some of the gymnogrammoid ferns have been evolved from primitive Osmundaceae (Bower, 1928, p. 74).

2. Schizaeaceae

Terrestrial ferns; *Lygodium* with dorsiventral protostelic creeping rhizome, in other genera solenostelic or erect and dictyostelic, scaly only in *Mohria*, in other genera hairy. Main branchings of frond dichotomous in *Schizaea*, unequally so in

Lygodium, pinnate in *Anemia* and *Mohria*; sporangia large, with apical annulus and lateral dehiscence, borne singly and marginal in origin, but apparently superficial when mature and each protected by a small flap; in *Schizaea* and *Lygodium* borne in close rows on pinnately arranged lobes, in *Anemia* on the reduced lamina of two specialized basal pinnae, in *Mohria* at vein-endings throughout the frond; spores tetrahedral or bilateral (*Schizaea* only).

The genera *Lygodium* and *Schizaea* are pantropic, *Anemia* only American and *Mohria* in South Africa. The four genera are very distinct, but undoubtedly allied. *Lygodium* has climbing (twining) fronds of indefinite growth in length, and peculiar branching, and so looks very different from *Schizaea*, but the first (non-scandent) leaves on a young *Lygodium* plant are branched exactly as in *Schizaea*, and in some species the leafy parts of the climbing frond are dichotomously branched also. It is interesting to note that *Lygodium*, with its specialized leaves, has the most primitive rhizome in the family.

This is also an ancient family, of which Jurassic and probably Carboniferous fossils are known. It is considered that some present-day ferns were evolved from ancestors which would be ranked as Schizaeaceae. The family are interesting as showing one way of evolution of superficial from marginal sporangia.

3. GLEICHENIACEAE

Rhizome creeping, rather slender, dorsiventral, externally hairy or scaly (scales with stiff marginal hairs); vascular system a protostele or solenostele. Fronds in most species large and deeply divided, in a few simply pinnate, often growing in stages by resting of the apical bud; ultimate leaflets with pinnate free veins. Sori of few sporangia developing simultaneously, on veins, on lower surface of the lamina. Sporangia rather large, with complete oblique annulus, opening by a longitudinal slit; spores tetrahedral.

Most authors have treated all members of this family as a single genus *Gleichenia*, or with separation of *Stromatopteris* and *Platyzoma*. In recent years, several authors have separated this large *Gleichenia* into its parts (which are well defined) and have ranked them as genera, as in Copeland (1947).

The most primitive frond form in the family (amply bi- or tri-pinnate in plan) is shown by *Hicriopteris*; this is reduced in *Gleichenia* proper, but essentially unchanged. In *Hicriopteris* the main axis of the frond ceases growth in length while each successive pair of pinnae develops. There is thus usually a dormant bud at the frond apex, but *no other dormant buds*. In both *Hicriopteris* and *Gleichenia* the rhizome is scaly, but the sori have very few large sporangia (a primitive condition).

In the genera *Dicranopteris* and *Sticherus*, the pinnae and axes of lesser order also cease growth and form dormant buds at their apices, but these buds remain dormant indefinitely, continued growth being by the preceding pair of lateral axes. This process reaches its most complex development in *Dicranopteris*, which is the most tropical of all the genera. It is notable that in *Dicranopteris* we have the most highly

developed frond form (and also the most highly developed sori) on plants which have the most primitive rhizome (protostelic and hairy) in the family. (It should be noted that the term *Dicranopteris* is here used in the restricted sense of Christensen (1938) and Copeland (1947), not that of Bower in *The Ferns*, Vol. II, 1926, ch. XXIV.)

4. MATONIACEAE

Rhizome creeping, hairy, with concentric solenosteles; fronds erect and dichotomously branched with pinnatifid branches in *Matonia*, pendulous with *Lygodium*-like branching in *Phanerosorus*; veins free or anastomosing near the costae; sori superficial, at a junction of several veins (*Matonia*) or terminal on small veins (*Phanerosorus*), of few large sporangia opening simultaneously, covered with an umbrella-shaped indusium; annulus incomplete, oblique; spores tetrahedral.

In the dichotomous main branchings of the frond, *Matonia* agrees with *Dipteris* and *Schizaea*; *Phanerosorus* resembles *Lygodium* in frond form. The sori are superficial, as in *Dipteris* and *Gleichenia*, but, unlike both, they are indusiate. A close alliance between *Dipteris* and *Matonia* is generally considered probable, on account of superficial sori and dichotomous main branchings of the frond, but the common ancestor must have been remote; fossil *Dipteris* and *Matonia* allies are distinct in Jurassic rocks. An alliance with *Gleichenia*, based on superficial sori and form of sporangia, is again probable but even more remote. Matoniaceae are now confined to Malay, and only occur in specialized habitats; fossils show that their ancestors must have been world-wide in distribution.

5. HYMENOPHYLLACEAE

Rhizome usually slender and long-creeping with distant fronds (in a few species short), the young parts covered with hairs, sometimes rootless. Fronds of many shapes, from very small and simple to fairly large and deeply dissected, the ultimate divisions always small, in most cases one-veined; lamina one cell in thickness except for the veins. Sori terminal on the ultimate one-veined lobes, or marginal at vein-endings on leaflets with many veins; receptacle more or less elongated, its base enclosed in the tubular or conical hollow base of the indusium, the apical part of the indusium more or less dilated, often more or less deeply divided into two lips; sporangia with oblique annulus; spores tetrahedral.

GENERA: *Hymenophyllum* and *Trichomanes* (the latter sometimes much subdivided); about 650 species, mostly pantropic and southern.

The largest species of *Trichomanes* have ample fronds to 60 cm. long or more, deeply divided, with one vein in each ultimate segment; the fronds of the smallest species, which must be considered reduced and specialized, are less than 1 cm. long. The receptacle of the sorus in many species continues to grow at the base, bearing new sporangia below the old, but in some species of *Hymenophyllum* the receptacle is short and bears few sporangia all developing simultaneously. The line of distinction between the old genera *Hymenophyllum* and *Trichomanes* is not sharp, which is one reason for subdividing them.

Plants of this family are well known as filmy ferns; most of them only grow in almost permanently very moist places, but some can withstand a certain amount of drought, to which they respond by curling up their fronds.

6. PLAGIOGYRIACEAE

Rhizome erect, short, rather stout, bearing a rosette of fronds, apex not scaly nor conspicuously hairy; vascular system a dictyostele. Stipes wide and flat at the base with a ridge down the back, bearing a row of wart-like aerophores on each side of the ridge; when young covered with mucilage secreted by hairs. Fronds simply pinnate, dimorphous, the fertile fronds on longer stipes than the sterile and with much narrower pinnae; pinnae quite separate or more or less broadly joined by a wing on either side of the rachis; veins free; fertile pinnae covered beneath with sporangia except for the midrib and edges, the sporangia protected when young by the reflexed edges; sporangia attached to the once-forked veins and on the surface near the veins; annulus complete, oblique, opening laterally.

Plagiogyria is the only genus; it occurs in both tropical Asia and in America; in Malaya only in poor acid peaty soils near the crests of mountain ridges. Bower suggests a relationship to Osmundaceae, and this seems quite possible. The broad base of the stipe may be a relic of the stipular structure found in *Osmunda*; and it may be significant that very young fronds of both *Osmunda javanica* and *Plagiogyria* are covered with mucilage. Superficially *Plagiogyria* is much like *Blechnum* (§*Lomaria*), but lacks scales.

7. DICKSONIACEAE

Tree-ferns with tall stout trunks, or rather large ferns with short prostrate stock bearing a tuft of fronds at its apex; trunk or stock thickly clothed with hairs. Fronds large, amply dissected (usually tripinnatifid to tripinnate), with free veins. Sori terminal on the veins, marginal, protected by an indusium consisting of two concave flaps, one from the upper side and one from the lower (or indusium cup-shaped); sporangia developing in gradate sequence, annulus oblique; spores tetrahedral.

GENERA: *Dicksonia*, *Cibotium*, *Cystodium*, *Thyrsopteris*.

A family of about thirty species, mainly tropical and southern in distribution. These ferns are a parallel group to Cyatheaceae, but retain strictly marginal sori and hairy stems, both primitive characters. Christensen (1938) also included in this family the genus *Culcita*, which is here placed in Dennstaedtiaceae; it agrees in vegetative form with *Dennstaedtia*, but in sori more with *Dicksonia*.

8. PROTOCYATHEACEAE

(*a*) *Lophosoria:* stock erect, bearing spirally arranged large bipinnate fronds, and at the base of each an abaxial bud which may develop into a solenostelic runner; apex of stock and frond covered with hairs; sori naked, of few large sporangia opening simultaneously, seated on a vein as in *Gleichenia*; annulus oblique, interrupted by a lateral stomium.

(*b*) *Metaxya:* rhizome creeping, hairy, with abaxial buds at leaf-bases; leaves simply pinnate with large pinnae and free veins; sori naked, irregularly placed on the veins, numerous, each with many sporangia simultaneously opening, and paraphyses; annulus nearly vertical.

Each of these two genera is represented by a single tropical American species. They agree in superficial sori and hairy rhizome, and are somewhat intermediate between Cyatheaceae and Gleicheniaceae.

9. Cyatheaceae

Stock erect, forming a massive trunk in most species, when old covered with a thick mass of interlacing roots; apex of trunk covered more or less densely with scales. Stipes scaly at the base, at least when young, bases of scales often raised and sometimes forming spines. Fronds large, usually bipinnate and more or less deeply tripinnatifid, in some species simply pinnate or even simple, more or less scaly on lower surfaces of rachises and costae; costules of pinna-lobes nearly at right angles to the costae, their veins strictly pinnate, veinlets simple or forked. Sori on the veins (never terminal), the sporangia attached to a small round receptacle, often mixed with hairs, without indusium or with a thin cup-shaped indusium which completely encloses the sorus when young; sporangia with a complete oblique annulus; spores tetrahedral.

A family of some 800 species, pantropic and southern. It was formerly the custom to recognize genera based on presence or absence of an indusium, but these genera are quite artificial. Groups of species based on other characters (scales, etc.) have for the most part not yet been successfully established.

Cyatheaceae have superficial sori, and are thought to be related to Gleicheniaceae. At least they must have descended from some similar ferns which had developed superficial sori at an early period. They are by far the largest group of tree-ferns, apparently still in active evolution, with many local species, especially on mountains in the tropics.

10. Polypodiaceae

Rhizome creeping, usually epiphytic, containing (as seen in section) a ring of small vascular strands, or a solenostele in a few primitive genera, externally scaly; scales peltate at the base (hairs present only in *Dipteris* and *Cheiropleuria*). Fronds two-ranked, on the upper side of the rhizome and nearly always jointed to it, simple and entire, or more or less deeply lobed, or pinnate; veins reticulate with free veins in the aeroles or rarely all free. Sori without indusia (though sometimes protected when young by peltate paraphyses), *either* almost round, small or large, sometimes sunk in cavities in the surface of the frond, on free or connected veins, or on a plexus of veins; *or* elongated parallel to the main veins; *or* elongated parallel to the margin; *or* acrostichoid on part or all of the frond. Spores without perispore, bilateral (spherical in some species of *Loxogramme*).

The following groups of genera may be recognized:

(*a*) The primitive genera *Dipteris* and *Cheiropleuria*, with terrestrial solenostelic

bristly rhizome, dichotomously branched frond (or main veins of the frond) with elaborate reticulation of the smaller veins.

(*b*) Genera with stellate hairs on the frond, the fronds dichotomously branched in *Platycerium*, rarely in the other genera: *Platycerium*, *Pyrrosia* (*Cyclophorus*), *Drymoglossum*.

(*c*) Genera with peltate paraphyses covering sori; fronds pedately branched in *Neocheiropteris*, otherwise simple: *Neocheiropteris*, *Pleopeltis*, *Lepisorus*, *Lemmaphyllum*, *Hymenolepis*.

(*d*) *Microsorium*, with simple or pinnately lobed fronds and many small sori lacking peltate paraphyses; leading (i) by lateral fusion of sori to *Colysis* and *Leptochilus* (acrostichoid), and (ii) by special development of base of frond to *Drynaria* and its allies.

(*e*) *Phymatodes*, with rather coriaceous fronds and evenly spaced round sori, leading (i) by lateral fusion of sori to *Selliguea*, (ii) by reduction to the small acrostichoid genera *Pycnoloma* and *Grammatopteridium*, (iii) by specialization of ant-inhabited rhizome to *Lecanopteris*, (iv) by production of pinnate fronds and modification of venation to *Polypodium*, in which the most reduced and specialized forms, including *P. vulgare*, have free veins.

It is to be noted that all the above, except those with elongate and acrostichoid sori, were included in *Polypodium* by Hooker. Elongation and fusion of sori have clearly occurred on several evolutionary lines, in some cases along with vegetative specialization. In *Platycerium* and *Drynaria* we have two of the most highly evolved groups of epiphytes; and the myrmecophilous *Lecanopteris* is scarcely less remarkable.

In this family, the most primitive known members have a pedate-dichotomous main branching of the frond, seen also in Mesozoic fossils, with a highly developed and peculiar reticulate venation. These ferns must have been derived from ancestors with more deeply dissected fronds and free veins. The only other living fern which has such fronds is *Matonia*; this has indusiate sori, and whether the ancestors of *Dipteris* had such, or how their sori became superficial, we have no evidence.

It seems fairly clear that the ferns of this family which have pinnately lobed or pinnate fronds are derived secondarily from simple-fronded ancestors, and that those with reduced fronds and free veins are at the limit of their evolutionary lines, not primitive. In all other main groups of ferns (as envisaged in the present scheme) there are highly dissected primitive frond-forms with free veins, and those with simple fronds and reticulate veins are the most highly evolved.

The genus *Loxogramme* presents a difficulty. Though agreeing with the polypodioid ferns in vascular anatomy of rhizome and stipe, in scales, and nearly with *Colysis* in venation (areolae less regular), it differs from all others of the family (except *Dipteris* and *Cheiropleuria*) in non-articulation of the fronds, and the spores of some species are almost spherical, apparently tetrahedral in origin, though other species have bilateral spores like those of other polypodioid genera. Copeland

associates *Loxogramme* with the grammitoid ferns, but I suggest that it is much more probably an aberrant member of the present family.

11. Grammitidaceae

Rhizome usually epiphytic, creeping or more or less ascending, in most cases (always?) dorsiventral in structure, with stipes alternating in two close ranks; vascular system a simple or somewhat dissected solenostele; scales usually brown, concolorous, cell-walls all uniform (superficial and lateral), base more or less peltate, edges often with stiff unicellular bristles. Stipes jointed to rhizome or not, in some cases (all?) with a single vascular strand; stipes and fronds usually bearing short or long stiff unicellular hairs or multicellular hairs with slender unicellular outgrowths. Fronds either simple or more or less deeply pinnatifid to pinnate, rarely to bipinnatifid; veins free or in simple fronds with occasional anastomosis, usually simply pinnate in the pinnae of pinnate fronds. Sori round, or more or less elongate along the veins, without indusia, superficial or more or less immersed in cavities which are sometimes extended into grooves. Sporangia naked or bearing stiff unicellular hairs; spores tetrahedral.

Genera: *Grammitis*, *Ctenopteris*, *Prosaptia*, *Calymmodon*, *Acrosorus*, *Xiphopteris*, *Cochlidium*, *Scleroglossum*, *Nematopteris*.

The genera are all nearly allied, and authors differ in the number they recognize. These ferns have usually been included in Polypodiaceae, and all except those with peculiar sori (*Scleroglossum*, etc.) in *Polypodium*, even in subgenus *Eu-polypodium*. They differ, however, from all (or almost all) truly polypodioid ferns in their spores, in their scales and hairs, and in their much simpler vascular anatomy. They are a pantropic, mainly epiphytic group, of some 250 species, nearly all small, and to be regarded as highly reduced and specialized. The most primitive vegetatively (only a few species of *Ctenopteris*) have highly dissected but still small fronds. I suggest for this group an origin from Gleicheniaceae or nearly allied ferns.

12. Thelypteridaceae

Rhizome creeping or suberect, not dorsiventral in structure but bearing fronds and roots on all sides; vascular system a modified solenostele or dictyostele. Scales usually narrow, bearing stiff or glandular unicellular hairs on edges and surfaces. Stipes containing two vascular strands at the base, these uniting upwards to a single strand U-shaped in section. Fronds usually pinnate-bipinnatifid, sometimes bipinnate, always much longer than wide, the lowest pinnae never much larger than the next and sometimes reduced; pinnae usually at a broad angle to the rachis, rarely very unequal (asymmetric) at the base; costules at a broad angle to the costa, veins in each lobe pinnate, veinlets usually simple but sometimes forked; a short or long translucent membrane usually present at the base of a sinus between two lobes, the veinlets of adjacent groups often joining the membrane, or joining to form an excurrent vein which meets the membrane; or where the pinna is hardly lobed

veinlets of adjacent groups meeting and forming free or anastomosing excurrent veins; unicellular stiff hairs, and sometimes glandular hairs, present on surfaces of rachis and lamina, variously distributed. Sori usually dorsal on the veins, round, with a reniform indusium (sometimes very reduced) or no indusium; in the latter case sometimes spreading a little along the veins, and the sporangia often setose. Sporangia usually bearing glandular hairs on their stalks, and sometimes glandular cells or setae on their surface. Spores bilateral or rarely tetrahedral, with a winged perispore, or the wings more or less broken up into small laminae or spines.

GENERA: *Thelypteris*, *Cyclosorus*, *Goniopteris*, *Meniscium*, *Glaphyropteris*, *Steiropteris*.

These ferns have usually been closely associated with *Dryopteris* (in Christensen's *Index Filicum* and most subsequent publications they have been included in *Dryopteris*). Christensen pointed out their distinctness from *Dryopteris*, and to his detailed studies of the group (1912) our present knowledge is largely due. *Thelypteris* agrees with *Dryopteris* in form of sori and spores, and differs in all other important characters. The arguments showing affinity between *Dryopteris* and the dennstaedtioid ferns do not apply to *Thelypteris*; I have suggested (1947, p. 130) an origin of *Thelypteris* from *Gleicheniaceae* or some primitive allied source.

Those species with broad pinnae have developed a distinctive form of anastomosing venation otherwise only seen in some species of *Athyrium* (*Diplazium*), but in *Athyrium* it is not quite identical. The Thelypteroid species in question have been placed in a distinct genus *Cyclosorus* on account of the anastomosis of veins; but a generic distinction on this character is probably unnatural. It is fairly clear that anastomosis has occurred on several evolutionary lines, i.e. that a natural division of the *Thelypteris* group should be based on other characters than venation. One character which might be used is the presence of many reduced lower pinnae with a more or less developed aerophore at the base of each. But at present such a natural division has not been worked out, and the genus *Cyclosorus* is convenient. The sori of some species have lost their indusia (in other cases there is a minute indusium, only seen at an early stage of development of the sorus). In such cases the sporangia often bear stiff unicellular hairs rather like those on the surface of the frond; an exactly similar development is found in Grammitidaceae.

13. DENNSTAEDTIACEAE

This family is divided into eleven very diverse subfamilies. There appears to me to be evidence for linking all of them with a primitive type something like *Dennstaedtia*, but in each the ultimate members are so divergent that they appear to have little in common. It is thus not possible to give a summary of the principal characters of the family, but such a summary is given for each subfamily. As indicated below, it seems possible that the subfamily Lindsayoideae, having simpler vascular structure than *Dennstaedtia*, may be more remotely related than the rest of the subfamilies, and that it should more naturally constitute a separate family.

Subfamily (i) DENNSTAEDTIOIDEAE

Rhizome slender, creeping, solenostelic, covered with hairs (except in *Orthiopteris* which has an erect stock with scales instead of hairs to protect the growing apex). Vascular strand in stipe more or less U-shaped. Fronds amply dissected (rarely simply pinnate), the ultimate leaflets with very asymmetric base. Sori terminal on the veins, with cup-shaped or 2-lipped indusium, or the lower lip lacking, or one lip fused with the lamina of the frond, the other forming a more-or-less pouch-shaped indusium. Sporangia in the more primitive genera gradately arranged, with oblique annulus, in others 'mixed', with almost vertical annulus. Spores tetrahedral.

GENERA: *Dennstaedtia*, *Microlepia*, *Leptolepia*, *Oenotrichia*, *Saccoloma* (*Ormoloma*?), *Orthiopteris* (including *Ithycaulon*), *Hypolepis*, *Culcita*, *Monachosorum*.

This group of genera are unquestionably primitive, and on the whole show only small developments from the marginal primitive position of the sorus. *Orthiopteris* is distinct in its erect stock with scaly covering, but its fronds are hardly different from *Dennstaedtia*. *Monachosorum* has also an erect stock. *Dennstaedtia* itself, as pointed out by Copeland (1947, p. 51), includes at least two distinct subgroups, and I am by no means sure that the line of distinction between *Dennstaedtia* and *Hypolepis* is sharp (*Hypolepis* is said to lack an inner indusial flap, but at least one species referred to it has such a flap). *Microlepia* is sorally the most advanced genus, but some species called *Microlepia* are very near *Dennstaedtia* in soral form. The species of *Dennstaedtia* and *Microlepia* need much more individual examination. Few of them have been examined in the details of soral development. Probably more variety exists than is at present known.

It seems fairly certain that Dicksoniaceae, which are massive hairy tree-ferns with rather primitive marginal sori protected by two flaps, are an offshoot from the same original stock as the dennstaedtioid ferns; but I regard Dicksoniaceae as a specialized distinct group which has not contributed further to fern evolution.

I suggest that the main lines of fern evolution from the primitive *Dennstaedtia* type have been:

(*a*) By way of something like *Microlepia* to *Davallia* (subfamily iii) and so to *Asplenium* (subfamily vi) and to *Lomariopsis* (subfamily viii) and possibly to *Blechnum* (subfamily vii); probably also to *Nephrolepis* (subfamily iv).

(*b*) By way of something like *Hypolepis* to the *Pteris* subfamily (v).

(*c*) To *Dryopteris* (subfamily ix) and *Athyrium* (subfamily xi).

(*d*) To the *Tectaria* subfamily (x).

These lines of development from a dennstaedtioid type seem to me fairly satisfactory except those of *Blechnum* and *Nephrolepis*. On all lines the extreme members (sometimes acrostichoid) are highly modified, but the less modified members show indications of their origins.

Subfamily (ii) LINDSAYOIDEAE

Mainly terrestrial; a few climbers or epiphytes. Rhizome slender, covered with bristles or rather narrow castaneous scales (not peltate at the base), vascular system

usually a modified protostele; stipes not jointed to the rhizome. Fronds amply divided to simple, leaflets in some cases dimidiate; veins free in the more amply divided forms, sometimes anastomosing in the less divided; midribs of leaflets sunk, the groove running into the rachis-groove, the thickened edge of the lamina decurrent on the edge of the rachis-groove. Sori nearly marginal, terminal on the veins, either singly or those on adjacent veins joining laterally; indusia attached basally and opening towards the leaf-margin, their sides sometimes attached also, the margin of the frond also thin and indusium-like, or like the rest of the lamina. Spores tetrahedral, without perispore.

GENERA: *Lindsaya*, *Tapeinidium*, *Sphenomeris* (or *Stenoloma*), *Odontosoria*, *Isoloma*, *Schizoloma*.

The (vegetatively) most primitive member of the subfamily is *Odontosoria*, which agrees in habit with the scrambling species of *Dennstaedtia* and *Hypolepis*, but is simpler anatomically. All members of the subfamily appear to have a closely similar primitive vascular system. Some have developed scales in place of hairs, but none have such highly developed scales as are found in most other subfamilies. Sorally there has been an advanced development of fusion-sori (all stages are seen within the genus *Lindsaya*).

This subfamily probably represents an independent development, parallel to that of Dennstaedtioideae, from the same more primitive stock. But the Lindsayoid ferns seem not to be on a main evolutionary line, and it might be better to place them in a separate family of their own. The genus *Lindsaya* shows a considerable degree of reduction in vegetative size; some species are specially adapted to peculiar habitats, such as river banks in the flood zone, and some are even epiphytes. The limits of the genera *Lindsaya*, *Sphenomeris* and *Schizoloma* are not easy to define.

Subfamily (iii) DAVALLIOIDEAE

Mostly epiphytes. Rhizome creeping, scaly, the scales in most cases with a peltate base; vascular system of rhizome as seen in transverse section consisting of broad dorsal and ventral steles with a variable number of small strands on either side. Stipes in two ranks, jointed at the base, with several separate vascular strands (except *Leucostegia*), the two largest uniting upwards. Fronds amply dissected, in a few pinnate or simple, often broadly deltoid, ultimate leaflets usually unequal at the base; veins free; midrib of leaflets raised, edge of lamina decurrent on to the edge of the winged pinna-rachis. Sori terminal on the veins, close to the margin, always single (never joined laterally), with indusium attached at the base and often also along the sides. Spores bilateral, without perispore.

GENERA: *Davallia* (including *Scyphularia*, *Parasorus* and *Trogostolon*), *Humata*, *Araiostegia*, *Davallodes*, *Leucostegia*, *Rumohra* (*sensu stricto* not of Ching and Copeland).

All genera except *Leucostegia* are closely related, and their limits are not always clear. Earlier efforts to define genera strictly on soral form led to a most unnatural arrangement; among species showing great similarity in every other way, there is

much variation in the shape and method of attachment of the indusium. The genus *Rumohra* (confined to the original species *R. adiantiforme*) is vegetatively very similar to *Davallia*, but has sori with peltate indusia.

The most primitive vegetative form in the family is shown by the more dissected species of *Araiostegia* (formerly often included in *Leucostegia*). *Leucostegia* shows a more primitive vascular structure than any other genus, and it shows some resemblances to *Lindsaya*; but in the peculiar anatomy of the stipe it agrees with *Davallia*.

The vascular structure of a *Davallia* rhizome is described as a dictyostele by Copeland, but it is very different from the dictyostele of a short erect radially symmetrical stock such as that of *Dryopteris*, which is usually cited as the type of a dictyostele. The *Davallia* rhizome is creeping and dorsiventral in structure, with remote leaves so that the leaf-gaps do not overlap. The stele is dissected, but its form is that of a solenostele with gaps which are mostly not leaf-gaps, and its roots are all associated with the broad ventral stele.

Subfamily (iv) OLEANDROIDEAE

This subfamily includes three very different genera, all highly specialized, and they may not be very closely allied.

(*a*) *Nephrolepis*. Stem short, erect, dictyostelic, bearing a rosette of fronds, bearing also slender horizontal runners which have at first a protostelic structure, but develop leaf-bearing stems at their tips; stems covered with peltate-based scales. Fronds not jointed at the base, simply pinnate (bipinnate in abnormal cultivated forms), with articulate pinnae and free veins; sori terminal, sometimes each on a small marginal lobe, with reniform to semicircular indusia, one species with a fusion-sorus.

(*b*) *Oleandra*. Stem scaly, erect and branched like a bush, or creeping and less branched; fronds borne in close groups, jointed near the base, simple, with free veins; sori in a close row on each side of the midrib, seated on the veins, shaped as in *Nephrolepis*.

(*c*) *Arthropteris*. Stem slender, creeping, scaly, bearing articulated fronds in two ranks; fronds much as in *Nephrolepis*; sori naked or with reniform indusia, terminal on the veins. *Diellia* and *Psammisorus*, with anastomosing veins, are possibly allied.

A prototype of *Nephrolepis* may possibly be seen in a fern called *Microlepia hookeriana* (Wall.) Presl, or *Scypholepia hookeriana* J.Sm. This has a solenostelic creeping hairy rhizome, and simply pinnate fronds with articulate pinnae and sori on marginal lobes as in some *Nephrolepis* species, but pouch-shaped as in *Microlepia*. This may not be an actual ancestral type; but it is a type intermediate between *Microlepia* and *Nephrolepis*, and indicates a way in which *Nephrolepis* could have evolved. Such an ancestral type could also give rise to *Arthropteris*. The highly dissected fronds found in some cultivated *Nephrolepis* are never found in nature, but they are probably a reversion to a more primitive vegetative condition and may be of value in indicating the primitive leaf-form from which the more reduced simply pinnate *Nephrolepis* evolved.

Oleandra is much reduced and specialized in the form of its simple entire fronds. The position of the sori near the midrib and not terminal on the veins may be due to the particular way in which webbing of the lamina has been evolved. Another remarkable feature of *Oleandra* is its habit of growth and the branching of the rhizome or stem, which in *O. neriiformis* forms bushy thickets on mountain ridges and forest edges in Malaya. It is possible that if the leaf-bearing stem of a *Nephrolepis* were extended and the runners shortened, something like *Oleandra* might result; but the precise morphology of *Oleandra* needs further study. It is, however, possible that *Oleandra* represents the end of a line of evolution quite distinct from that of *Nephrolepis*, and that similarity of soral form and scales is accidental.

Subfamily (v) PTERIDOIDEAE

Rhizome creeping and hairy in the primitive members, scaly and radial in structure in others (high-climbing in *Stenochlaena*); fronds amply dissected in the more primitive members, more or less reduced (to simply pinnate) in others; more amply divided fronds with grooved rachis-branches and grooved costae of leaflets, the edge of a costa-groove decurrent upon the edge of the groove of the rachis which bears it; veins mostly free, uniting in one or more series of areoles, without included free veinlets, in some genera; sori mainly fusion-sori, with or without abortion of the inner indusium, acrostichoid in *Acrostichum* and *Stenochlaena*.

GENERA: *Pteridium*, *Paesia*, *Lonchitis*, *Anisosorus*, *Histiopteris*, *Pteris* and near allies; *Neurocallis*, *Acrostichum*, *Stenochlaena*.

The members of this subfamily have been rather fully discussed by Bower (1928, ch. XXXVIII). He does not, however, mention the characters of grooved rachises and costae, which seem to me significant, and does not include *Stenochlaena*. *Stenochlaena* is indeed rather isolated, but in complex anatomy it resembles *Acrostichum*, and also in glands at the bases of the pinnae, and in aspect of the living fronds; the edges of the narrow fertile pinnae of *Stenochlaena* are thin and lack sporangia, resembling somewhat the outer indusium (frond-edge) of *Pteris*.

Subfamily (vi) ASPLENIOIDEAE

Rhizome in a minority creeping or climbing and dorsiventral, usually suberect or erect and radial in structure; vascular system a dictyostele; scales peltate at the base, clathrate or nearly so. Stipes containing two vascular strands at the base, joining upwards to form a single strand with four arms (more or less X-shaped). Fronds amply dissected to simple, rarely dimorphous; veins free except in a few of the less dissected forms. Sori linear along one side of a vein, in nearly all cases with a narrow indusium opening towards the costa of the leaflet which carries it, or in a lobed leaflet towards the costule of the lobe, or in some simply pinnate or simple fronds a less regular arrangement, sometimes a sorus on both sides of a vein. Spores bilateral, with perispore.

GENERA: *Asplenium*, *Phyllitis*, *Diplora*, *Camptosorus*, *Ceterach*, *Pleurosorus*, *Pleurosoriopsis* (?).

Asplenium and *Athyrium* have often been (at least in part) confused, and have sometimes been united because of the similarity of their sori, but their distinctness is now generally recognized. I regard *Asplenium* as having originated from ferns something like *Davallia*; the section *Loxoscaphe* of *Asplenium* was formerly united with *Davallia*, and shows a more or less intermediate soral condition. Most species of *Asplenium*, however, have a short stock with radially symmetric dictyostelic structure, and at the same time a simpler vascular anatomy of the stipe which retains resemblances to *Davallia* in the way the two strands unite.

Asplenium shows all stages from large deeply dissected fronds to species with simple fronds; and the reduction has occurred on several independent lines, so that one cannot, for example, group all *Asplenium* species with simple fronds in one subgenus. Spore characters are helpful in recognizing natural groups within *Asplenium*. Some of the epiphytic species show peculiar structures of the lamina which indicate how they have been evolved from more highly dissected species. Little anastomosis of veins has occurred in this group of ferns, though some species with simple fronds have a very broad lamina (e.g. *A. nidus* L.).

In the course of webbing of the lamina, some soral modifications have occurred, notably in some veins bearing a sorus on each side (on one side only in all the more dissected species), or on adjacent sides of two veins close together. The condition of a sorus on each side of a vein is similar to that shown by the more advanced species of *Athyrium* (those usually called *Diplazium*).

Subfamily (vii) BLECHNOIDEAE

Terrestrial ferns with stout scaly erect rhizome. Stipes containing several vascular strands. Fronds pinnate or bipinnate; veins free or with some anastomosis. Sori short or long, on veins or vascular commissures parallel to the midrib of a pinna, in a single line, or (in *Doodia*) in one to three rows on each side, protected by a marginal indusium (if the fertile pinna is narrow) or a superficial, intramarginal to subcostal exterior and introrse membranous indusium. Spores bilateral, with or without perispore.

GENERA: *Blechnum*, *Sadleria*, *Woodwardia*, *Doodia*, *Brainea*.

Bower places the section *Lomaria* of *Blechnum* as primitive in this subfamily, deriving from it *Eu-Blechnum* with its broad fertile pinnae; and from *Eu-Blechnum* he derives *Phyllitis*. I have argued that this is an unnatural and improbable sequence.

The most primitive frond form in the subfamily is seen in *Woodwardia*, which has large deeply bipinnatifid fronds, and this genus may well show the most primitive sorus form also. The sori of *Woodwardia* are short, on the inner side of veins forming subcostal areoles. Such a condition could be derived by anastomosis of veins bearing *Asplenium*-like sori. *Blechnum indicum* has simply pinnate fronds, and the distal parts of the pinnae bear *Woodwardia*-like sori. These sori have no special vascular supply other than the veins bearing them. In *Blechnum orientale* the sorus-bearing veins have become partly modified and do not appear externally

as normal veins. In *B.* (*Lomaria*) *vestitum* the vascular supply to the broader sorus has developed into a broad band of tracheids close to the surface quite distinct from, though having contact with, the normal veins. This series indicates a possible mode of origin of the *Lomaria* condition.

It should be noted that the blechnoid ferns are nearly all terrestrial, with rather massive erect rootstock, and have several vascular strands in the stipe, which gives no indication of a relationship to *Asplenium*. Copeland suggests an origin of *Blechnum* from *Athyrium* (*Diplazium*). *Athyrium* is a genus of terrestrial ferns with short radially constructed rootstocks; it perhaps presents a more likely origin than *Asplenium* for the blechnoid ferns. The matter needs more study. If any *Blechnum* could be induced to produce copiously bipinnate fronds like the cultivated *Nephrolepis*, that might give useful evidence.

Subfamily (viii) LOMARIOPSIDOIDEAE

Rhizome creeping or climbing or epiphytic, dorsiventral, with a broad basal vascular strand which supplies the roots and one or more dorsal strands (the leaves in two or more ranks according to the number of dorsal strands); stipes jointed to the rhizome or more usually not, containing several separate vascular strands; scales peltate, small or large, those on stipe and frond smaller than on rhizome; no unicellular hairs. Fronds simple, simply pinnate or bipinnate, the pinnae in climbing and epiphytic genera jointed to the rachis, terminal unjointed lamina present or not; distinctive bathyphylls, usually more dissected than the acrophylls, present in some climbing genera; veins free, or uniting near the margin, or in several series of areoles with or without free excurrent included veins. Fertile fronds with reduced lamina, covered beneath with sporangia (except in *Thysanosoria*), a special vascular supply to the sporangia developed or not. Spores with perispore, except in *Lomagramma*.

GENERA: *Egenolfia*, *Bolbitis*, *Lomariopsis*, *Thysanosoria*, *Teratophyllum*, *Lomagramma*, *Elaphoglossum*.

Of the genera here assembled, *Egenolfia* was formerly united with *Polybotrya* (a dryopteroid genus), *Bolbitis* with *Leptochilus* (Polypodiaceae), *Lomariopsis* and *Teratophyllum* were much confused and united with *Stenochlaena* (here placed in Pteroideae, by Copeland in Blechnaceae), *Lomagramma* was also associated with *Leptochilus*, and *Elaphoglossum* has always been given a rather isolated position. Christensen (1938) united all except *Elaphoglossum* as 'acrostichoid genera of probably Dryopteroid origin'.

The distinctive common characters are the dorsiventral rhizome with its broad root-bearing ventral vascular strand, the acrostichoid condition, in most cases the simply pinnate fronds with free veins, and the perispore.

The primitive leaf-form of the subfamily is probably indicated by the bathyphylls of *Teratophyllum*, which are bipinnate. In *T. wilkesianum* and allied species, the bathyphylls are large, and not unlike the *Davallia* subfamily in aspect, except for the articulation of pinnae and pinnules. The only member of the subfamily which

shows any indication of separate sori is *Thysanosoria*; here the sori are terminal on the veins, on small marginal lobes of the lamina. Together with the dorsiventral structure of the rhizome, and the shape of the bathyphylls of *Teratophyllum*, this is a good indication of an origin from the dennstaedtioid ferns, probably on a line near *Davallia*, which has also a ventral root-bearing stele and peltate scales. But the *Lomariopsis* subfamily do not show the joining of the large stipe-bundles as in *Davallia*. The rachis of all members of the subfamily is winged, and the edge of the lamina of pinnae is decurrent on the wing (unless the pinnae are jointed) as in *Davallia*.

The genera of the subfamily may be divided into three groups. (1) *Egenolfia* and *Bolbitis* with short-creeping rhizomes, usually growing on rocks in shady forest, sometimes climbing short distances up tree-trunks but never high-climbing. (2) *Lomariopsis*, *Thysanosoria*, *Teratophyllum*, *Lomagramma* with high-climbing rhizome usually starting life on rocks or the bases of trees or the forest floor, and always rooted in the ground. (3) *Elaphoglossum*, always epiphytic or on rocks, with rather short, not high-climbing rhizome. The rhizome-habit thus divides the genera biologically into three distinct groups, each with a different set of environmental conditions to which it must be adapted. These conditions have doubtless been the cause of leaf-differences. The creeping rock-ferns, usually growing beside streams, have the most uniform environment, and have in most cases no articulation of pinnae or fronds. The high-climbing ferns, rooted in the earth, their fronds far from the water supply, may seasonally suffer from dry conditions, and all have pinnae jointed to the rachis, their fronds on the whole being fairly thin in texture (few if any grow in places exposed to the sun). *Elaphoglossum*, with simple fronds, has these jointed to the rhizome, and the fronds are usually fleshy or leathery, except in those species which only grow in moist shady places.

Subfamily (ix) DRYOPTERIDOIDEAE

Terrestrial ferns with (in all except a small minority) erect or suberect rhizomes of radial structure with fully developed dictyostele; stipes with several vascular strands; scales without stiff unicellular hairs, marginal teeth when present formed of two adjacent cells. Fronds amply divided with free veins (except *Cyrtomium*), leaflets with unequal base; costae of leaflets grooved, the edge of the groove decurrent on a similar interrupted groove in the pinna-rachis, edge of lamina decurrent on side of rachis. Sori round, usually with indusium; indusium basally attached and round to reniform, or peltate, or in a few cases globose or nearly so; spores bilateral, with perispore.

GENERA: *Woodsia*, *Stenolepia*, *Acrophorus*, *Diacalpe*, *Peranema*, *Lithostegia*, *Dryopteris*, *Polystichum*, *Polystichopsis*, *Cyrtomium*, *Didymochlaena*, *Polybotrya*, *Maxonia* (?), *Gymnocarpium* (?).

There is general agreement that these genera are allied, and that those at the beginning of the list show most primitive characters. Most authors have associated these more primitive genera with Cyatheaceae. I have suggested (1947, p. 150)

that there is better evidence for associating them with a dennstaedtoid source. The evidence of prothalli, of the 'sorus-genus' *Deparia* Hk. & Grev. and of rachis-characters, all point to *Dennstaedtia* rather than *Cyathea* as a prototype. The more primitive genera bear coarse multicellular hairs on the upper surface of veins and on the margins of costae, costules and rachises. Similar hairs occur in some members of the *Athyrium* and *Tectaria* subfamilies, which are considered to have had a parallel origin. *Dryopteris*, *Polystichum* and allied genera have hairless fronds. *Didymochlaena* is a peculiar pantropic genus with little difference among its species; its leaflets are strikingly like *Lindsaya* in some ways, but not in sori. Its position in the present subfamily is probably correct, but its relation to the other genera is neither near nor precisely definable. *Gymnocarpium* was placed by Christensen (1938) with *Thelypteris*, but it seems more likely to belong here, though its creeping rhizome with 2-ranked fronds is peculiar. It needs further investigation.

Subfamily (x) TECTARIOIDEAE

Rhizome erect or suberect, with complex dictyostele; stipes not jointed to it, containing several vascular strands. Fronds amply divided to simple, either narrow in outline, or deltoid with basiscopically enlarged basal pinnae; costules and costae raised, not grooved; multicellular hairs usually present on upper surface of rachis, costae and other parts of frond; veins free or variously anastomosing. Sori round with reniform indusia, terminal or dorsal on the veins; or without indusia, in which case they may spread along the veins; or acrostichoid.

GENERA: (*a*) *Ctenitis*, *Lastreopsis*, *Tectaria*, *Heterogonium*, *Quercifilix*, *Stenosemia*, *Cyclopeltis*, *Hemigramma*, *Amphiblestra*, *Pleuroderris*, *Dictyoxiphium;* (*b*) *Pteridys*, *Pleocnemia*, *Arcypteris*.

The basic genus of this subfamily is *Ctenitis*, which in variation of frond form and in sori matches *Dryopteris* closely, but which differs constantly from *Dryopteris* in raised costae bearing many short multicellular hairs. I suggest that the two had a common origin (along with *Athyrium*) in the primitive *Dennstaedtia* stock, the ancestral form having grooved hairy costae (as in some existing species of *Dennstaedtia*), the *Dryopteris* tribe retaining the grooved costae but (except in the primitive genera) losing the hairs, *Ctenitis* losing the groove but retaining the hairs.

Tectaria is linked with *Ctenitis* by some species with free veins which were included in a separate genus *Ctenitopsis* by Ching, but they are so near *Tectaria devexa* that a separation seems unjustified. These primitive species of *Tectaria* have broader, fewer pinnae than *Ctenitis*, and the basal basiscopic vein of each group springs directly from the costa, not from the costule which bears the other veins. The majority of species of *Tectaria* have anastomosing veins, the extent and complexity of anastomosis progressing according to the broadening and simplification of the parts of the lamina, culminating in simply pinnate and simple-fronded species such as *T. polymorpha* and *T. singaporensis*. In these species the venation pattern closely matches that of the broad-leaved members of Polypodiaceae (*sensu stricto*), a remarkable case of convergent evolution. Some exindusiate species of *Tectaria*

have even been referred to *Polypodium*, but the erect rhizome, non-articulate stipes and spores distinguish them.

Lastreopsis and *Heterogonium* are clearly related to *Ctenitis*. *Lastreopsis* has a creeping rhizome and broadly deltoid fronds. *Heterogonium* has narrow fronds, and shows stages to a distinctive form of anastomosis of veins, also to an acrostichoid condition.

Quercifilix and *Stenosemia* are acrostichoid genera of one species each, both derived from *Tectaria*, but evidently from different species of *Tectaria*, so that they must either be kept as separate genera or both merged in *Tectaria* (the same problem arises regarding the offshoots of the Polypodiaceae with peculiar fusion-sori).

Dictyoxiphium is a monotypic tropical American genus which has long been associated with *Lindsaya* on account of its almost continuous marginal sorus, with indusium as in *Lindsaya*. Its alliance with *Tectaria* is proved by erect habit, scales, venation and spores. *Pleuroderris* and *Amphiblestra* are allied to *Dictyoxiphium*, but show a soral condition somewhat nearer to *Tectaria*. *Cyclopeltis* is in many ways peculiar, and its affinities are uncertain.

Pteridrys, *Pleocnemia* and *Arcypteris* form a distinct subgroup, characterized by the presence of prominent teeth (pointing out of the plane of the frond) in the sinuses between the pinna-lobes. These teeth give the living plants a very distinctive appearance, hardly seen in dried herbarium specimens. A vein always passes to each sinus-tooth. The teeth are probably relics of an ancestral character in the *Dennstaedtia* stock. Somewhat similar teeth occur in *Egenolfia*, and they are indicated in *Dryopteris sparsa* and other species. In *Pteridrys* the teeth are sharper and more prominent than in the other two genera of the subgroup. The veins in *Pteridrys* are free, a primitive character, and one of the Chinese species is quite a large fern, of size similar to *Arcypteris*.

Pleocnemia was founded by Presl on its venation only, for which reason Beddome and others added to the original species several of *Tectaria* which are not nearly allied but have veins uniting in a single series of costal areoles. As limited to the original species and its near allies, *Pleocnemia* is a very distinct genus, in its very narrow rhizome-scales, glandular hairs and sinus-teeth. *Arcypteris* is nearly allied to *Pleocnemia* and the two genera might well be united.

Subfamily (xi) ATHYRIOIDEAE

Rhizome terrestrial, erect or suberect, of radial symmetry; stipes with two vascular strands at the base, uniting upwards to a single U- or V-shaped strand. Scales with marginal teeth formed of two adjacent cells. Fronds highly dissected to simple; veins nearly always free, or in a few species the veinlets of adjacent groups uniting to form parallelogram-shaped areoles; rachises and costae more or less scaly, hairless or the upper surface sometimes with thick multicellular hairs; costules and costae grooved, the edge of the groove decurrent upon the edge of a similar rachis-groove, the lamina decurrent on the side of the rachis. Sori usually

linear on the veins, with linear indusia, in some species reniform or J-shaped, in *Cystopteris* round with basal indusium. Spores with perispore.

GENERA: *Athyrium* (including *Diplazium*), *Cystopteris*.

The more dissected species of *Athyrium* are hardly different in frond form from *Dryopteris*, but the anatomy of the stipe is simpler and more like that of *Thelypteris*, for which reason some authors have associated *Thelypteris* and *Athyrium*. The scales of the two genera are, however, very different.

The primitive sorus form in this genus is supposed to have had a reniform indusium; the double sorus then developed by elongation along both sides of the vein and a break in the indusium at the cross-over (distal end of sorus). The double sorus of *Athyrium* is thus considered to have had a different origin from that of some species of *Asplenium*.

The largest species of *Athyrium* (usually called *Diplazium*) are very large ferns, almost tree-ferns, but with very short trunk, with fronds as large as any *Cyathea*. They are an extremely polymorphic group, evidently successful, mainly found as terrestrial ferns in primitive shady tropical forest. Few species of *Athyrium* have developed anastomosis of veins. In *A. esculentum* the anastomosis is rather like that of members of Thelypteridaceae but not quite identical. In the broader lamina of *A. accedens* (sometimes placed in a separate genus *Callipteris*) the arrangement is different.

14. ADIANTACEAE

In this family I unite the gymnogrammoid and vittarioid ferns of Bower, who gives evidence for associating them; the alliance, however, is not at all close. The name Adiantaceae was chosen for this particular grouping because *Adiantum* is the largest and most distinctive genus of the family so formed.

(*a*) *Gymnogrammoid ferns.* These are an extremely varied and specialized group, mostly of dry climates and more or less xerophytic and reduced vegetatively. Bower (1928, ch. XXXIX) discusses their soral forms, which in some cases show developments parallel to the pteroid ferns (with which Copeland associates them in the family Pteridaceae). These ferns need much more careful comparative examination in the living state, especially as regards soral development, scales, etc.

In addition to the genera included by Bower, *Taenitis* should also be placed here. In spite of Bower and Goebel, I cannot think there is any doubt of a close alliance between *Syngramma* and *Taenitis*; in fact, it is quite probable that they should be united as one genus. Copeland places *Syngramma* with *Taenitis* among the lindsayoid ferns (in Pteridaceae).

One of the least xerophytic of the gymnogrammoid ferns is *Coniogramme*. Copeland states: '*Coniogramme* is the gymnogrammoid derivative of *Pteris*; in the parent genus, its origin is in the group of *P. cretica*, in which such species as *P. insignis* and *P. pellucida*, in their juvenile stages, are effectively indistinguishable from neighbouring young plants of *Coniogramme*.' I have not seen young plants of *Coniogramme*, but I believe they would show significant distinctions from *Pteris*. My own view of *Coniogramme*, independently arrived at, was expressed as follows

(1947, p. 157): 'the peculiar raised and grooved costae, with their edges decurrent on grooved rachises, of *Pteris* and the more primitive genera related to it, are not matched in the present group of genera; not even in *Coniogramme*, which has the most primitive frond form and might be expected to retain such characters.... I would agree with Bower that *Coniogramme* probably originated from a stock allied to the Schizaeaceae, and that the *Dennstaedtia-Pteris* series had also a similar origin, but on a separate line of descent from an early stage.'

(*b*) *Vittarioid ferns.* Epiphytes with densely hairy roots, short rhizome covered with dark clathrate scales and tufted, usually limply pendulous simple fronds (dichotomously lobed in *Hecistopteris*). Veins reticulate except in *Hecistopteris* and in the very reduced genus *Monogramme*; in *Vittaria* with a single row of areoles each side of the costa and a continuous submarginal vein formed of the outer margins of the areoles; sori along the veins, more or less immersed; paraphyses present.

The genus *Hecistopteris*, which like all members of the group is very reduced, is the only member showing lobing of the fronds; the lobing is almost regularly dichotomous, and the branching of the free veins similar.

VII. SUMMARY

Intensive morphological studies have been devoted to the more primitive ferns, which represent a small minority of living species, but too little is yet known about the vast majority of other ferns, with the result that recent attempts at a natural classification show considerable differences of treatment.

The problem is complicated by convergent evolution in the characters of almost all parts of a fern plant. Not only similar soral form, but also similar frond form, types of venation, scales, etc. have been developed on different evolutionary lines.

To illustrate the nature of the problem an attempt has been made to state the probable characters of a primitive leptosporangiate fern, and the kinds of ways in which existing ferns have developed from this condition. Evolutionary change in different parts of the plant has proceeded in different ways and to different degrees in the many genera of existing ferns. Primitive characters of one kind or another are shown by a great number of ferns, along with highly advanced characters of other kinds.

Recent schemes of classification are briefly compared, and a summary is given of the author's own scheme, with notes on evolutionary trends in the various groups as he sees them.

Much more information is needed on which to establish a really satisfactory scheme. The present one is put forward in the hope that others will take up the work. With modern facilities for travel, it is to be hoped that more botanists will come to the tropics and see ferns and other too-little-known plants in their native habitats. Morphological study needs to be undertaken with an understanding of the living plant and of its environment.

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STRUCTURE AND FUNCTION OF NEURONES IN RELATION TO MENTAL ACTIVITY

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I. INTRODUCTION

Structure and function of protoplasm are intimately correlated with each other. Any function is accompanied by regular changes of the structure of protoplasm on the microscopical, submicroscopical and stereochemical levels. The brain is the organ of mind and therefore our mental activities are also intimately associated with the structure and function of the protoplasm of the neurones of the cerebral cortex. During the development of an embryo various components of undifferentiated cells are gradually transformed into the corresponding components of the neurones. The activities of neurones are to be regarded as modified and more developed functions of undifferentiated cells. This has been stressed particularly by Lillie (1932) in his book, *Protoplasmic action and nervous action.* For this reason any progress in investigations dealing with the structure and function of undifferentiated cells must be of importance for understanding the activities of neurones.

Different life phenomena have frequently a common foundation, and therefore comparative investigations contribute greatly to the solution of biological problems. Moreover, single cells, such as sea-urchin eggs, are in many respects more suitable for experimental treatment than neurones which are components of complicated tissues, and therefore investigations on the former cells have in some cases contributed more to the solution of certain common problems than investigations performed on the latter cells. The purpose of this article is to show to what extent recent investigations on undifferentiated cells contribute to the understanding of the functioning of neurones concerned with our mental activities.

II. COMPARISON BETWEEN NEURONES AND UNDIFFERENTIATED CELLS

Large neurones are, in several respects, similar to oocytes. This fact has been noticed by numerous cytologists. The nuclei of large neurones are similar to germinal vesicles. The chromatin of both, neurones and oocytes, exhibits a strong affinity to

acid dyes; it is essentially an 'oxychromatin' (Heidenhain, 1907) poor in nucleic acid. On the other hand, large amounts of ribonucleic acid are present in the cytoplasm of oocytes and neurones, which fact indicates that rapid protein synthesis proceeds in both kinds of cells (Caspersson, 1941; Hydén 1943, 1947). Moreover, there is a striking similarity between the pathological structural changes of the cytoplasm of neurones (Bielschovsky, 1932; De Castro, 1932) and the pathological structural changes of the cytoplasm of sea-urchin eggs (Runnström, 1928*c*, *d*; Monné, 1945, 1947; Runnström & Monné, 1945*a*, *b*). It will be demonstrated below that neurones and sea-urchin eggs are also similar in several other respects.

The macromolecules, microsomes (Claud, 1946) or cytochondria (Opie, 1947) extracted from the cytoplasm of various cells by recent cytochemists are identical with the Nissl substance or the chromidia investigated much earlier in fixed preparations by numerous cytologists. It is unfortunate that as yet there is no commonly accepted terminology. Richard Hertwig and his pupils, particularly Goldschmidt, have recognized that chromidia are a general component of the cell, and I have therefore proposed (1948) to retain the excellent term 'chromidia' coined by Hertwig and to drop inadequate or new denominations. The chromidia contain proteins, ribonucleic acid, lipoids, calcium, magnesium, and, according to several cytochemists, also respiratory and hydrolysing enzymes. In this connexion it is of interest to note that the chromidia of neurones were regarded by Marinesco as centres of cellular oxidations as early as in 1919.

Investigations on sea-urchin eggs by means of the polarizing microscope, initiated by Runnström, continued by Moore and Miller, and by Schmidt, and further developed by myself definitively prove that the cytoplasm has a fibrillar structure. The ground cytoplasm is a texture of fibrils whose thickness has been estimated to about 50–100 mμ. Moreover, I have provided experimental evidence that chromidia are integral components of cytoplasmic fibrils. These fibrils consist of chromidia containing ribonucleic acid and interchromidia free from ribonucleic acid, which regularly alternate with each other. In this respect the cytoplasmic fibrils are very similar to chromosomes. The latter consist of chromomeres containing thymonucleic acid and interchromomeres free from thymonucleic acid, which also regularly alternate with each other. For details and literature concerning this subject the reader is referred to a recent review (Monné, 1948).

The chromidia of neurones are also known in the literature under the name of Nissl substance. The minute chromidia are either uniformly scattered throughout the cytoplasm or are aggregated into clusters of variable size. Two kinds of fibrils are known to be present within the cytoplasm of neurones: neurofibrils and cytoplasmic fibrils. The texture of cytoplasmic fibrils is known in literature as spongioplasm. The staining properties of neurofibrils and of nucleic-acid-containing cellular components are entirely different from one another. Thus, there is no evidence that neurofibrils contain any considerable amount of nucleic acid. Moreover, no investigator has observed that chromidial substance is associated with neurofibrils. On the other hand, the question has been discussed as to whether or not the minute chromidia are

attached to cytoplasmic (spongioplasmic) fibrils. Some authors claim really to have observed chromidia connected with the cytoplasmic fibrils (Legendre, 1908; Erhard, 1912; Kunze, 1921). In the case of sea-urchin eggs it has been shown that the cytoplasmic fibrils consist of ribonucleic-acid-containing chromidia and ribonucleic-acid-free interchromidia, which regularly alternate with each other, and therefore there is no reason to believe that this is otherwise in the case of neurones.

The question has been debated whether the neuroplasm (cytoplasmic fibrils) or the neurofibrils are of primary importance for the conduction of nerve impulses and in general for the function of neurones. The former hypothesis seems to be true. This is supported by the fact that the chromidia, which are considered to contain enzymes, are not associated with the neurofibrils, but with the neuroplasm only. The neurofibrils seem merely to constitute the skeleton of the cell. In addition to this they may play some auxiliary role in nerve conduction.

I have no doubt that the 'neurotubules' extracted from various nerves by de Robertis & Schmitt (1948) correspond to neurofibrils. These neurotubules or neurofibrils exhibit a cross-striation under the electron microscope. They consist of regularly alternating sections which either strongly adsorb or do not adsorb phosphotungstic acid. It is probable that the strongly adsorbing sections consist chiefly of basic amino-acid residues. This conclusion is supported by the fact that phosphotungstic acid is employed in histological technique as an important mordant in the staining process of basic proteins.

III. STIMULATION AND INHIBITION OF PHYSIOLOGICAL AND MENTAL ACTIVITIES

Runnström (1928*c*, *d*) and I (Monné, 1947) have showed that the structure of the cytoplasm of sea-urchin eggs coarsens under the influence of narcotics. A similar coarsening was observed when respiration of the eggs was inhibited by potassium cyanide or sodium azide (Runnström, 1928*b*, 1930; Monné, 1947). From these facts Runnström inferred that energy derived from respiration is necessary for the maintenance of the normal structure of cytoplasm. These structural changes are reversible within certain limits. Irreversible and excessive coarsening of the cytoplasmic structure causes death. It is a well-known fact that warm-blooded animals die rapidly when their respiration ceases. This means that the neurones of these animals are particularly sensitive to lack of oxygen. Death occurs because excessive and irreversible coarsening of the cytoplasmic structure is more rapidly brought about in neurones than in any other cell when respiration is inhibited. The same phenomenon may also be produced under the influence of narcotics.

Sea-urchin eggs whose cytoplasmic structure has become coarser under the influence of experimental agents exhibit a striped or spotted appearance in fixed and stained preparations, just like the resting neurones of warm-blooded animals which have not been subjected to any experimental treatment. The ground cytoplasm is a texture of fibrils. Agglutination of these fibrils is the essential feature of the coarsening of the cytoplasmic structure. The number of lateral linkages between

the fibrils is increased and therefore coarse cytoplasmic nodes are formed. As stated above, the cytoplasmic fibrils consist of the ribonucleic-acid-containing chromidia and the ribonucleic-acid-free interchromidia, which regularly alternate with each other. The chromidia, which are intensely stainable with basic dyes, aggregate with each other simultaneously with the agglutination of the cytoplasmic fibrils. The chromidia are amassed within the above-mentioned cytoplasmic nodes. Large, deeply stained clusters of aggregated chromidia are formed and therefore the cytoplasm becomes striped or spotted when its structure coarsens.

It is improbable that the cytoplasm of living uninjured neurones exhibits such a coarse structure. On the contrary, it is very probable that the structure of the cytoplasm is relatively fine and that it coarsens rapidly prior to fixation because neurones are particularly sensitive to lack of oxygen and because it is necessary to kill or to narcotize the animals whose tissues are excised. Narcosis and inhibition of respiration at the moment of death must produce a rapid coarsening of the cytoplasmic structure in highly sensitive cells. Moreover, this coarsening may well be promoted by the alcoholic fixatives which are used with preference for the demonstration of Nissl substance. Thus, it is probable that the cytoplasm of resting neurones has a much finer structure when living and uninjured than in the fixed condition. The cytoplasmic fibrils and the chromidia of living uninjured neurones in the resting condition are presumably only weakly agglutinated together. The fibrils of these cells are as a rule situated relatively far away from one another.

Mature unfertilized and fertilized sea-urchin eggs differ greatly from each other. Numerous facts, chiefly accumulated by Runnström and his associates (Runnström 1928*b*, *c*, 1930; Runnström & Monné, 1945*a*, *b*; Monné, 1947; Öhman, 1944; and others) clearly prove that the physiological, chemical, physico-chemical (colloidal), physical and structural properties of mature unfertilized and of fertilized sea-urchin eggs are very different. Runnström (1928*c*, *d*) and Öhman (1944) demonstrated that the condition of the lipoids is changed upon fertilization, which fact is of particular importance because lipoids are concerned with the irritability of the cell. Finally, it must be added that the rate of respiration of sea-urchin eggs is strongly increased upon fertilization, a fact which has been known since the time of Warburg's (1908) investigations. It is obvious that fertilized sea-urchin eggs are in a strongly stimulated condition.

Many facts found in the literature show that resting and stimulated neurones are also very different from each other. There is strong evidence that mature unfertilized eggs of sea-urchins are comparable to resting neurones, and that fertilized eggs of these animals are comparable to stimulated neurones. Thus, the differences between unfertilized and fertilized sea-urchin eggs must be similar to the differences between resting and stimulated neurones. It may be assumed that mental activities are also depressed when the cytoplasm of neurones is in a condition similar to that of the cytoplasm of mature unfertilized sea-urchin eggs and that mental activities are stimulated when the cytoplasm of the neurones is in a condition like that of the cytoplasm of fertilized sea-urchin eggs.

Clear differences can be demonstrated not only between resting and excessively stimulated neurones, but also between depressed and weakly stimulated ones. In order to prove this latter statement the reader is referred to two excellent figures by Ramón y Cayal reproduced by Cowdry (1932) in Penfield's book, vol. 1, p. 14. One of these figures represents a neurone taken from a fully active animal, and the second is one from a hibernating animal. The difference in structure is quite evident. The cytoplasmic structure of the neurones in the active animal is fine. On the contrary, the structure of the cytoplasm in the neurones of the hibernating animal is coarse, evidently because this animal is physiologically in a depressed condition. A similar, but much less pronounced coarsening of the cytoplasmic structure may also occur during ordinary sleep.

These facts are in perfect harmony with the investigations by Runnström (1928*c*, *d*) and by myself (Monné, 1947) on sea-urchin eggs. It was found that the structure of the cytoplasm coarsens when the physiological activity of these eggs is depressed by narcotics. Moreover, it has been demonstrated that under the influence of weakly hypertonic sea-water coarsening of the cytoplasmic structure is more easily produced in mature unfertilized eggs than in experimentally activated eggs (Runnström & Monné, 1945*b*). Evidently, the cytoplasmic colloids are less hydrated in the former eggs, which are in a depressed condition, than in the latter, which are in a stimulated condition. Thus, the structure of the cytoplasm is coarse and can easily be made still coarser under the influence of experimental agents when the eggs are in a depressed condition. On the contrary the structure of the cytoplasm is fine, and it is more difficult to make it coarse under the influence of experimental agents, when the eggs are in a stimulated condition. It is very probable that mental activities are also stimulated when the structure of the cytoplasm of the neurones is fine, and that they are depressed when this structure is coarse. Not only in the above-mentioned cases, but also in many other cells coarsening of the cytoplasmic structure is a sign of decreased activity. The so-called ergastoplasm is formed when the structure of the cytoplasm coarsens. This ergastoplasm has been observed in a great variety of cells and it has been regarded by many earlier cytologists as an indication of depressed or pathological conditions (Lutz, 1921; Herzig, 1934).

In fixed and stained preparations the cytoplasm of excessively stimulated neurones has an entirely different appearance from that of resting neurones. This fact has been well known since the time of Nissl's investigations. This excessive stimulation may be brought about under the influence of various physical and chemical agents; it may be due to injury such as resection of axons and it may be produced under the influence of muscular and probably also mental overstrain. Chromatolysis is the term commonly used in the literature to denote the condition of the cytoplasm in excessively stimulated neurones.

It has been mentioned above that resting neurones have a spotted or striped appearance in fixed and stained preparations. Moreover, it has been demonstrated that this appearance is due to coarsening of the cytoplasmic structure, which occurs prior to fixation and which is increased under the influence of the fixatives employed.

In these neurones the Nissl substance or the chromidia are in an agglutinated condition. Excessively stimulated neurones do not exhibit a striped or spotted appearance in fixed preparations. In these neurones chromatolysis takes place, which means that the Nissl substance or the chromidia are not in an agglutinated, but on the contrary in a dispersed or 'pulverized' condition. The minute chromidia of these neurones are uniformly scattered throughout the whole cytoplasm. The ground cytoplasm is a texture of fibrils. As stated above, these fibrils consist of the ribonucleic-acid-containing chromidia and the ribonucleic-acid-free interchromidia, which regularly alternate with one another. In excessively stimulated neurones the chromidia do not aggregate because the cytoplasmic fibrils do not join each other. The cytoplasmic structure does not coarsen, prior to fixation, when excessively stimulated neurones are excised from the animals. Evidently, in excessively stimulated neurones coarsening of the cytoplasmic structure cannot be produced as easily and as rapidly as in resting neurones. The condition of the cytoplasm is profoundly changed upon stimulation. All intermediate stages between resting and excessively stimulated neurones may be observed.

Marinesco (1918) and Bielschovsky (1932) arrived at the conclusion that cytoplasmic colloids are more hydrated in stimulated than in resting neurones. Obviously, the cytoplasmic fibrils of stimulated neurones are strongly hydrated, and therefore the structure of the cytoplasm does not coarsen so rapidly when the animals are killed. The result mentioned is in harmony with the investigations of Runnström (1928*c*, *d*), who found that the cytoplasmic colloids are more hydrated in fertilized than in unfertilized sea-urchin eggs. It is well known that the former are in a stimulated and the latter in a depressed condition. The above-mentioned conclusion is also supported by the experiments of Runnström & Monné (1945*b*), who demonstrated that under the influence of weakly hypertonic sea water the structure of the cytoplasm coarsens more easily in unfertilized than in experimentally activated eggs. Thus, mature unfertilized sea-urchin eggs are comparable to resting neurones and fertilized eggs to stimulated neurones.

Runnström (1928*c*, *d*) found that the structural changes in the cytoplasm of mature sea-urchin eggs brought about by hypertonic sea water are reversed completely in the case of successful fertilization. This has later been confirmed by Runnström, Monné & Broman (1943) and by myself (Monné 1944, 1945). Under the influence of hypertonic sea water the structure of the cytoplasm of mature sea-urchin eggs coarsens, and intensely stainable clusters of aggregated chromidia are formed. Upon successful fertilization the structure of the cytoplasm becomes fine and the aggregated chromidia are dispersed, which phenomena are the essential features of chromatolysis. It is a well-known fact that sea-urchin eggs are stimulated at the moment of fertilization. Thus, in sea-urchin eggs chromatolysis is produced under the influence of stimulation exactly as in the case of neurones.

A similar effect is brought about in gland cells when elaboration of secretion-granules is stimulated. In these cells the ergastoplasm disappears (Pacaut & Vigier, 1905; Heidenhain, 1907; Lutz, 1921; Zimmermann, 1927), which fact means that

the structure of the cytoplasm becomes fine (see p. 301). The opposite phenomenon is brought about when secretion is inhibited: the structure of the cytoplasm becomes coarse and the ergastoplasm reappears. This ergastoplasm is particularly well developed during hibernation and starvation (Lutz, 1921). All these facts show that the cytoplasm of stimulated cells is different from the cytoplasm of depressed cells. Chromatolysis, stimulation and activation are more easily brought about when sea-urchin eggs are pre-treated with serum or serum proteins (Runnström, *et al.* 1943). In this connexion it is of interest to note that the composition of blood-plasma proteins is changed in various mental diseases (Ludlum de Witt, 1944). It is possible that the irritability of neurones is influenced (increased or decreased) by the changing composition of the blood-plasma proteins.

Phosphatase activity is strongly increased both upon excessive stimulation of neurones (Bodian & Mellors, 1945) and upon fertilization of sea-urchin eggs (Wicklund, 1948). Thus, in this respect too, stimulated neurones and stimulated (fertilized) sea-urchin eggs resemble each other. Nevertheless, acid phosphatase is activated in the former case and alkaline phosphatase in the latter. The photographs of Bodian & Mellors (1945) show that acid phosphatase is activated in the regions of the cell where the structure of the cytoplasm is fine and the chromidia are dispersed, while it is inactivated in regions where the structure of the cytoplasm is coarse and the chromidia are agglutinated.

The differences in the absorption of ultra-violet rays by the cytoplasm of resting and excessively stimulated neurones as detected by Hydén (1943, 1947) are chiefly due to condensation and loosening of the texture of cytoplasmic fibrils. The absorption is high when the structure of the cytoplasm is coarse and when consequently the chromidia are aggregated. The absorption is low when the structure of the cytoplasm is fine and when consequently the chromidia are dispersed. The facts reported by Bodian (1947) prove that the structure of the cytoplasm is fine in chromatolytic, that is to say in excessively stimulated neurones and coarse in resting neurones. Even in the case when ribonucleic acid was removed, coarse cytoplasmic nodes, consisting of proteins, were found to be absent from chromatolytic, but present in resting neurones. In the case of chromatolysis the chromidia are dispersed and therefore the absorption of ultra-violet light is low. The neurones of manic-depressives and of schizophrenics exhibit a low ultra-violet absorption (Hydén, 1947), no doubt because they are in the condition of chromatolysis. In the case of these mental diseases the neurones are in an excessively stimulated condition and therefore their colloidal properties are changed. Less severe chromatolysis may be the essential phenomenon underlying neuroses. Under pathological conditions the irritability of the neurones is impaired. Feelings are possibly associated with the physiological processes underlying stimulation and inhibition of the activities of the neurones. Thus, the differences described in ultra-violet absorption are chiefly due to differences in the colloidal condition of the cytoplasm of the neurones. Nevertheless, it may be that in the case of extreme stimulation decrease in ultra-violet absorption is also due, to some extent, to a real decrease in the amount of ribonucleic

acid and proteins present within the cytoplasm, as stressed by Hydén (1943, 1947). Malonitrile causes coarsening of the cytoplasmic structure and consequently also increased ultra-violet absorption. Changes in the colloidal properties of the cytoplasm are accompanied by changes in the irritability of the neurones.

What mechanism is responsible for the alterations of the cytoplasm in connexion with stimulation and inhibition of cellular activities? Protoplasm is generally considered to be a soft thixotropic gel whose physical state is subjected to continual and reversible changes under experimental and physiological conditions. Under certain conditions protoplasm is more solid, under others more liquid. Moreover, some regions of the protoplasm of a cell are more solid and others more liquid. It is evident that changes in the physical state are due to changes in the chemical properties of the protoplasm. Now, both chemical and physical properties of protoplasm are controlled by enzymes. Stimulating and inhibiting agents do not influence the properties of protoplasm directly: they influence primarily the enzyme systems which control the chemical and physical properties. The thixotropy of protoplasm is not a passive physical phenomenon, but rather an active biological thixotropy controlled by several antagonistic systems of substances, particularly enzymes. Some of these substances present within the cell tend to liquefy the protoplasm by breaking down fibrils and by dissolving the lateral linkages between these fibrils, while other substances in the cell tend to solidify the protoplasm by building up fibrils and by producing new lateral linkages. This is the reason why the structure of protoplasm cannot be seriously altered by means of micro-needles and why protoplasm exhibits simultaneously the properties of liquids and solids. The fibrils are reconstituted almost at the same instant as they are cut through. It is very probable that the solidifying agents are similar to enzymes and other substances operative in blood clotting. It has been stressed, particularly by Heilbrunn (1943), that this clotting system is present within the cell. Blood clotting may be brought about by proteolytic enzymes and therefore it is probable that the clotting of cytoplasm is also caused by these enzymes which are known to be present in any cell. This conclusion is supported by the fact that clotting phenomena of cytoplasm are rapidly induced by potassium cyanide (Monné, 1947) which is able to activate the proteolytic enzyme kathepsin (Bersin, 1940).

Clotting is solidification, and solidification is crystallization. Clotting of blood and clotting of cytoplasm are crystallization phenomena produced by enzymes. Crystallization of protoplasm does not proceed spontaneously, but it is induced by enzymes just like the chemical reactions which constitute cellular metabolism. Decrease in crystallinity means liquefaction and increase in crystallinity is solidification. The ground cytoplasm of living sea-urchin eggs not subjected to any experimental treatment is isotropic, because the birefringent cytoplasmic fibrils are oriented in all directions. Birefringence of the ground cytoplasm may appear or disappear under experimental conditions. In the former case the crystalline properties of the cytoplasm are increased and in the latter decreased. Birefringence of the ground cytoplasm appears when the cytoplasmic fibrils are to some extent regularly oriented.

This occurs when the number of lateral linkages between the cytoplasmic fibrils is increased under the influence of the clotting system activated by some experimental agent. This birefringence disappears when the cytoplasmic fibrils become scattered in a disorderly manner. This occurs when the number of lateral linkages between the cytoplasmic fibrils is decreased under the influence of the liquefying system activated by some experimental agent. Thus, the crystallinity of the ground cytoplasm is increased under the influence of the solidifying or clotting system and decreased under the influence of the liquefying system. Increase in crystallinity is not necessarily associated with increase in viscosity. On the contrary, liquefaction, that is to say decrease in crystallinity, may be associated with increase in viscosity. In this connexion it is of interest to note that glass is not regarded as solid, but as a highly viscid fluid, because it does not exhibit any crystalline properties.

The structure of cytoplasm coarsens, the chromidia aggregate with each other and the texture of cytoplasmic fibrils is condensed when the number of lateral linkages between these fibrils is increased under the influence of the clotting system. On the contrary, the structure of the cytoplasm becomes fine, the cytoplasmic texture is loosened, the cytoplasmic fibrils separate from each other, the chromidia are dispersed and consequently chromatolysis occurs when the number of lateral linkages between the fibrils is decreased under the influence of the liquefying system. It is probable that the number of water-attracting molecular groups of the cytoplasmic fibrils is decreased under the influence of the clotting system and increased under the influence of the liquefying system. This conclusion is supported by the fact that vacuoles appear when clotting of cytoplasm occurs (Monné, 1947) and that they disappear when this clotting is reversed. Evidently the cytoplasmic fibrils are dehydrated in the former case and rehydrated in the latter. Thus, it seems that the hydration of cytoplasm is also controlled by enzymes. These enzymes may be operative in the adaptation of the cell to changing osmotic conditions.

The rate of respiration is high when the structure of cytoplasm is fine and it is low when the structure of cytoplasm is coarse. The structure of cytoplasm becomes coarse under the influence of the clotting system and it becomes fine under the influence of the liquefying system. Consequently, it must be assumed that the rate of respiration is decreased when the former system is activated and that it is increased when the latter system is activated. Chantrenne (1944) found that the rate of respiration is strongly decreased when liver is subjected to centrifuging. No doubt this is due to coarsening of the cytoplasmic structure, which is also brought about by centrifugal treatment. This coarsening is probably produced by the clotting system which is activated under the influence of centrifuging.

The network of cytoplasmic fibrils is condensed in the animal region and loosened in the vegetative region of the egg (Monné, 1946*a*), probably because the clotting system prevails at the animal, and the liquefying system at the opposite pole. The antagonism between the animal and vegetative poles discovered by Runnström (1928*a*) may be due to the antagonism between the clotting and liquefying enzyme systems. It is probable that the rate of respiration of the single chromidium is

decreased in any region of the developing egg where the chromidia are densely packed as a consequence of the condensation of the cytoplasmic network, and that this rate of respiration is increased wherever the chromidia are dispersed as a consequence of the loosening of the cytoplasmic network.

Narcosis may be defined as any reversible inhibition of cellular activities. These activities only proceed normally when variations in the physical properties of protoplasm do not exceed certain limits. Since the time of Claude Bernard, semi-coagulation of protoplasm has been regarded to be the cause of narcosis. Bancroft and associates (1932) tried to show that narcosis is brought about by agents which coagulate the cytoplasmic colloids, and stimulation by other agents which disperse these colloids. It is highly probable that this semi-coagulation is not brought about directly by lipoid-soluble narcotics, but indirectly under the influence of the clotting system which is activated by these narcotics. In the case of sea-urchin eggs it is easy to show that the structure of the cytoplasm coarsens under the action of narcotics. At the same time birefringence of the ground cytoplasm may appear. This fact proves that crystallization, an essential feature of clotting, may be brought about by these narcotics. These clotting phenomena are reversible within certain limits, and are thus compatible with life. Heilbrunn (1927) and myself (Monné, 1947) have advanced the view that narcosis may also be due to excessive liquefaction of cytoplasm. Recovery of cytoplasm from excessive clotting or excessive liquefaction is brought about by subsequent activation of the enzyme system which produces the opposite effect.

Marinesco (1918) advanced the view that concussion of the brain acts on the enzymes of the neurones. It is probable that clotting of the cytoplasm of the neurones is produced by enzymes and other substances which are activated in the case of concussion. Ingvar (1923) arrived at the conclusion that concussion and centrifuging act similarly on the properties of the neurones. In this connexion it is of interest to note that coarsening of the cytoplasmic structure (Monné, 1944), probably under the influence of the clotting system, and decrease in the rate of respiration (Chantrenne, 1944), are produced by centrifuging. These are probably the essential phenomena underlying concussion of the brain. Chromatolysis seems to be a protective reaction against any injury by which the clotting system is activated. Chromatolysis means activation of the liquefying system which counteracts the solidifying effect of the clotting system. Thus, chromatolysis must lead to recovery from excessive clotting. This is supported by the fact that no sign of chromatolysis could be detected in the neurones of persons who have been instantaneously killed as a result of injury to the head. On the other hand, chromatolysis was found in the neurones of those who survived for some time after the concussion produced by an accident (Rand & Courville, 1946). Chromatolysis sets in several hours subsequent to injury, it attains its maximum after 15 days and then it is gradually reversed within 3–6 months (Bodian, 1947). Chromatolysis occurring subsequent to concussion is a process similar to activation of sea-urchin eggs subjected to shaking.

It has been mentioned above (p. 301) that the structure of the cytoplasm of neurones coarsens during hibernation and possibly also during ordinary sleep. Coarsening of the cytoplasmic structure is a clotting phenomenon. Thus, it seems that hibernation and ordinary sleep are brought about when the clotting system of the neurones is activated strongly in the former case and slightly in the latter. In the case of ordinary sleep the purpose of this clotting may be to counteract chromatolysis which would occur upon excessive stimulation. It is known that hibernation is brought about under the influence of hormones. From this fact it is inferred that clotting and liquefaction of the cytoplasm are also controlled by hormones.

The formation of the ergastoplasm in cells whose activity is depressed, particularly in glands during hibernation and starvation, is also a clotting phenomenon. This ergastoplasm decreases or disappears completely when secretion-granules are elaborated upon stimulation of glands by means of pilocarpine (Caspersson, Landström-Hydén & Aquilonius, 1941), which fact proves that the structure of the cytoplasm becomes fine under the influence of the liquefying system.

Under normal conditions a labile equilibrium between the liquefying and solidifying (clotting) systems is maintained. This equilibrium, which is slightly and reversibly disturbed under the influence of ordinary stimuli, may be subjected to rhythmical changes and oscillate within certain limits in adaptation to variations of environmental conditions. Cellular activities are controlled by these two antagonistic systems. Abnormal changes of the cellular medium may temporarily or definitively upset the equilibrium. In the former case narcosis is produced and in the latter cytolysis with ensuing death of the cell. Moderate liquefaction and moderate clotting (solidification) of the cytoplasm are normal physiological phenomena. This liquefaction and solidification may succeed each other in a certain rhythm. Instability of the structure of the cytoplasm with ensuing amoeboid movements may be due to a disturbed balance between the liquefying and clotting enzyme systems. Excessive, but still reversible, clotting or liquefaction of cytoplasm produces narcosis. By mobilizing the liquefying system the cell may adapt itself, within certain limits, to the action of physical and chemical agents (e.g. lipoid-soluble narcotics) which induce excessive clotting, and by mobilizing the clotting system the cell may adapt itself to the action of agents which induce excessive liquefaction of the cytoplasm.

Extreme, irreversible clotting or liquefaction brings about cytolysis and death of the cell. Extreme clotting leads to black cytolysis and extreme liquefaction to white cytolysis. The essential feature of any cytolysis is the separation of the lipoids from the proteins (Knaffl-Lenz, 1908; Loeb, 1913). In the case of black cytolysis this is accompanied by coagulation of the proteins, but not in the case of white cytolysis. Cytolysed cells become opaque ('black') when their proteins are coagulated. There exists a series of intermediate forms between these two kinds of cytolysis which have been known since the time of Loeb's (1913) investigations. Both forms of cytolysis have also been observed in the case of neurones and described as coagulation and liquefaction (Bielschovsky, 1932). Both extreme stimulation and extreme inhibition

may cause cytolysis. Extreme activation of the clotting system leads to black cytolysis and extreme activation of the liquefying system to white cytolysis. Heilbrunn (1943) advanced the view that clotting with ensuing death of the cells is produced under the influence of heat and lipoid-soluble narcotics. This clotting may be responsible for cell death also in the case when respiration is inhibited by potassium cyanide and sodium azide.

Chromatolysis is produced in the neurones of pantothenic-acid deficient animals (Follis & Wintrobe, 1945). This fact indicates that an enzyme which contains this acid is operative in the process by which chromatolysis, that is to say stimulation, is reversed. It may be that this enzyme is concerned with the clotting of cytoplasm.

There is no doubt that two antagonistic processes are released under the influence of any stimulus. At first one process is released which tends to produce a change and this is followed by another process which tends to reverse this change. It has been found by several investigators that under the influence of various stimulating agents the interior cytoplasm becomes at first more liquid and later more solid (Heilbrunn & Daugherty, 1933; Angerer, 1936, 1939; Heilbrunn, 1943). It is evident that at first the liquefying system is activated and later the solidifying or clotting system. Stimulation would cause cytolysis if these two antagonistic systems did not limit each other. Clotting is operative in the recovery process after the change produced by stimulation. In this connexion it is of interest to note that sea-urchin eggs are stimulated upon fertilization and that, according to Loeb (1913), two antagonistic agents (which produce and prevent cytolysis) are concerned with the activation of these eggs. Moreover, it has been emphasized by Runnström (1947) that a number of enzyme systems are activated upon fertilization. The clotting phenomena displayed by sea-urchin eggs have been particularly investigated by Heilbrunn (1943).

The changes of cytoplasm which occur upon inhibition or stimulation can be demonstrated with particular clarity when exaggerated under the influence of experimental agents. These phenomena can be directly observed when sea-urchin eggs, treated with hypertonic sea water, are fertilized. Successful fertilization is possible when these eggs are first treated with serum or serum proteins (Runnström *et al.* 1943). Typical clotting phenomena are brought about by hypertonic sea water. Thus, the structure of the cytoplasm coarsens, the cytoplasmic fibrils and the chromidia agglutinate and birefringence of the ground cytoplasm appears. Lipoid-soluble narcotics produce the same effects. All these clotting phenomena are gradually reversed when the eggs are stimulated to development at the moment of fertilization. Eggs treated with hypertonic sea water develop normally when the clotting is completely reversed. These facts disprove the hypothesis of Heilbrunn (1943) that clotting is the essential feature of stimulation. Moderate liquefaction is the essential feature of stimulation, and excessive clotting is the essential feature of inhibition produced by lipoid-soluble narcotics. Thus, clotting is not the essential feature of stimulation. Nevertheless, as mentioned above, moderate clotting is activated subsequent to stimulation in order to reverse the change produced by

stimulation. Moreover, it is possible that after stimulation the disturbed equilibrium between the liquefying and solidifying systems oscillates for some time in either direction before being definitively established. These oscillations may cause rhythmical changes in the condition of the cytoplasm. The irritability of the cell is permanently changed when moderate liquefaction or moderate clotting of the cytoplasm become permanent.

Oscillations of the equilibrium mentioned may explain the well-known fact that narcotics exert a stimulating effect in low and an inhibitory effect in high concentrations. The first effect of narcotics and stimulating agents is the same. Both produce liquefaction of the cytoplasm. This initial liquefaction is followed by slight clotting (strong enough to restitute the former condition of the cytoplasm) in the case of ordinary stimuli and by excessive clotting in the case of narcotics. Evidently the equilibrium between the liquefying and clotting systems is disturbed under the influence of lipoid-soluble narcotics, so that at first the former system is slightly and then the latter system is strongly activated. The initial stimulation is explained by the initial liquefaction of the cytoplasm, produced by enzymes.

Clotting, solidification or crystallization, that is to say an increase in the number of lateral linkages between the cytoplasmic fibrils, is the essential feature of inhibition, and liquefaction, that is a decrease in the number of lateral linkages between these fibrils, is the essential feature of stimulation. As mentioned above (p. 305), this liquefaction may be followed either by decrease or increase in viscosity. The latter phenomenon seems to occur upon fertilization of sea-urchin eggs. Nevertheless, I have some doubts as to whether it is possible to determine the viscosity of cytoplasm without changing it by the methods employed. Moreover, the 'viscosity' of cytoplasm is a specific property, not identical with, but only analogous to, the viscosity of simple lifeless fluids.

Lipoids are concerned with the irritability of cells (Lillie, 1924; Runnström, 1928*b*, 1930; Monné, 1946*b*, 1948). It is supposed that upon stimulation the lipoid-protein compounds of the cytoplasm are temporarily broken down. Irritability is suspended when the condition of the cytoplasm is changed so that the lipoid-protein compounds cannot be broken down under the influence of ordinary stimuli. Narcotics are known to be able to suspend the irritability of the cell. From this fact it is inferred that the strength of the linkages between lipoids and proteins is increased under the influence of narcotics. This may be due to activation of the clotting system. Weakening of these linkages may be due to activation of the liquefying system. At any rate, the cell is able to regulate the force by which the lipoids are bound to the proteins. This is illustrated by the well-known fact that cytolysis under the influence of various experimental agents is more easily produced in fertilized than in mature unfertilized sea-urchin eggs (Runnström, 1928*c*). The separation of lipoids from proteins is the essential feature of cytolysis. Thus, the above-mentioned fact proves that the separation of lipoids from proteins is more easily produced in fertilized eggs which are in a stimulated condition than in unfertilized eggs which are depressed.

It is well known that permeability is increased upon stimulation (Lillie, 1924, 1932). The cell becomes temporarily permeable to lipoid-insoluble substances. The following alterations of the plasma membrane may explain this increase in permeability. The plasma membrane consists of lipoid molecules and of protein fibrils. The former are oriented perpendicular to the surface of the cell and the latter tangential in all directions. Upon stimulation, the lipoid-protein compounds of the plasma membrane are broken down. The enzymes responsible for respiration and liquefaction of the cytoplasm are activated when this occurs. The latter enzymes dissolve the lateral linkages between the fibrils whereby the superficial cytoplasm is liquefied. This may facilitate the contraction of fibrils (folding or spiralization of polypeptide chains) under the influence of the energy released by means of the respiratory enzymes associated with these fibrils. These contractile movements may explain the active transfer of lipoid-insoluble substances across the plasma membrane. This hypothesis has been advanced by myself (Monné, 1946*b*, 1948) and by Libet (1947). During recovery from stimulation, liquefaction is followed by clotting of the superficial cytoplasm, whereupon all the changes described are reversed.

IV. PHYSIOLOGICAL PROCESSES ASSOCIATED WITH INTELLECTUAL ACTIVITIES

Hydén (1943) found that the cytoplasm of neurones is particularly rich in ribonucleic acid and that it rapidly synthesizes proteins. Oocytes, too, have a cytoplasm rich in ribonucleic acid and therefore they are able to elaborate large amounts of reserve proteins as yolk. Neurones do not contain and consequently do not elaborate any reserve proteins. Neurones very rapidly synthesize the proteins of the living substance itself. The proteins of the cytoplasmic fibrils are chiefly in question here. Rapid protein synthesis within the cytoplasmic fibrils seems to be the most outstanding feature of the activity of neurones. Hydén (1947) advanced the view that mental functions are correlated with the nucleoprotein metabolism of nerve cells.

Synthesis of new proteins within the cytoplasm of various cells is induced either by external or internal agents. During development of an embryo, synthesis of new proteins is brought about under the influence of internal agents, the nuclear genes. Nevertheless, the cells are also able to 'learn' to synthesize new proteins under the influence of external agents. Synthesis of new antibodies and enzymes is chiefly in question here. The macrophages, lymphocytes or plasma cells (Fagraeus, 1948) are able to 'learn' to synthesize new antibodies against new antigens. Both antibodies and antigens are proteins. The specificity of antibodies is a well-known fact. Thus, new proteins are synthesized under the influence of other proteins which penetrate the above-mentioned cells. Moreover, some cells, particularly yeast cells, are able to 'learn' to synthesize new enzymes in adaptation to new substrates (Linderström-Lang, 1940; Lindegren, 1945). In this case new proteins, the enzymes, are synthesized under the influence of relatively simple chemical compounds present in the medium of the cells. By synthesizing new enzymes the cells are able to 'learn' to induce new chemical reactions. It may be that neurones are particularly 'intelligent'

and consequently able to 'learn' with particular ease, to synthesize new proteins within their cytoplasmic fibrils. Only the amazing variability of proteins can explain the fact that all that we experience during our life is stored in our memory (Monné, 1948).

Chromomeres and chromidia are very similar to one another. The former are the seat of nuclear genes and the latter may be the seat of cytoplasmic genes (plasmagenes). It is supposed that the cytoplasmic genes are also able to mutate (Haddow, 1944). The cytoplasmic proteins are synthesized by the chromidia. Chromidial mutations causing synthesis of new specific proteins within the neurones may occur at any moment when new perceptions and concepts arise in the mind.

Ontogenetic development is a process of rapid structure formation. Proteins are the most important chemical constituents of all structural components of cells and tissues. For this reason protein synthesis means structure formation. The rapid structure formation during development is due to rapid protein synthesis. This rapid protein synthesis takes place because the cytoplasm of embryonic cells is very rich in ribonucleic acid (Caspersson, 1941; Brachet, 1945). Also neurones are very rich in ribonucleic acid and they rapidly synthesize proteins. Intense protein synthesis in the neurones is an indication of rapid structure formation within the nervous system, chiefly at the submicroscopical and chemical levels.

It is known that mental functions are intimately associated with physiological functions of the neurones of the cerebral cortex. Structure and function are intimately associated with one another. For this reason our mental functions must also be accompanied by regular structural alterations of the cytoplasm of the neurones at the microscopical, submicroscopical and chemical levels. It is obvious that memory must be associated with some permanent structural changes of the cytoplasm (cytoplasmic fibrils) of neurones. These postulated alterations are called engrams. Chromidial mutations, synthesis of new specific proteins and new connexions between various chromidia and between various neurones may be the structural changes of the brain associated with memory, thinking, and, in general, with all intellectual activities. Some alterations of this mechanism may be associated with clever and others with pathological thinking. It may be that a high mutation rate of the chromidia is characteristic of clever thinking. Instincts are inherited because the specific mode of protein synthesis and structure formation within the nervous system are also inherited. Inherited behaviour seems to be determined by protein synthesis within the nervous system under the influence of nuclear genes, and acquired behaviour is determined by protein synthesis within the nervous system under the influence of exterior agents. Feeling is possibly associated with changes in the colloidal properties of the lipoid-protein compounds of the cytoplasm (see p. 303).

The similarity between heredity and memory has been noticed by a number of biologists. The egg 'remembers' all the characters of the parents and therefore it is able to reproduce an organism essentially similar to that of the parents. These characters are recorded in the specific structures of the chromosomes, which are

transmitted from one generation to the other. In this connexion it is of interest to note that the chromosomes of all kinds of cells and the cytoplasm of neurones are particularly rich in nucleic acid which is concerned with protein synthesis. Chromosomes are the seat of hereditary factors, the cytoplasm of neurones the seat of memory. Thus, heredity and memory must be connected with intense protein synthesis.

V. SUMMARY

1. The properties, structural, physiological and colloidal, of the cytoplasm of resting and stimulated neurones differ greatly. In this respect resting neurones are similar to mature unfertilized sea-urchin eggs, which are in a depressed condition, and strongly stimulated neurones resemble fertilized sea-urchin eggs, which are in a highly stimulated condition. The importance of this comparison for understanding the functioning of neurones is stressed.

2. The ground substance of cytoplasm is a texture of fibrils. These fibrils consist of ribonucleic-acid-containing chromidia and ribonucleic-acid-free interchromidia, which regularly alternate with one another. The structure of cytoplasm is coarse when the fibrils are agglutinated together. The chromidia, which are intensely stainable with basic dyes, aggregate with each other simultaneously with the agglutination of the cytoplasmic fibrils. The structure of cytoplasm is fine when the fibrils are separated from each other and the chromidia are consequently dispersed.

3. The structure of the cytoplasm coarsens rapidly when resting neurones of warm-blooded animals are excised. This change does not occur when strongly stimulated neurones are excised. Coarse cytoplasmic structure and the aggregated condition of chromidia are correlated with decrease in the rate of respiration, narcosis, hibernation, sleep, and in general with inhibition of cellular activities. Fine cytoplasmic structure and the dispersed condition of chromidia are the characteristic features of chromatolysis, which occurs upon stimulation.

4. Solidifying (clotting) and liquefying enzyme systems control the properties of cytoplasm. The former enzyme system is responsible for the coarsening of cytoplasmic structure and may be activated by narcotics. Stimulation and chromatolysis are brought about when the latter enzyme system is activated. It is probable that clotting enzymes are activated in case of concussion of the brain, and liquefying enzymes upon recovery.

5. It is suggested that mental activities are depressed when the structure of the cytoplasm of neurones is coarse and that these activities are increased when this structure becomes fine.

6. Synthesis of new specific proteins may be the mechanism underlying memory. The structural changes in cytoplasm associated with thinking are probably due to protein synthesis.

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BIOCHEMICAL ASPECTS OF MAMMARY GLAND FUNCTION

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I. INTRODUCTION

The intermediary metabolism of the lactating mammary gland is a comparatively unexplored by-way of biochemistry as compared, for instance, with muscle metabolism. Muscle is a tissue *par excellence* for biochemical investigation since in its case energy-yielding chemical transformations can most easily be related to work done. By contrast, secretory glands such as the mamma, whose function is synthesis, present much more serious difficulties in this respect, and it is not surprising that as yet much less is known about the intermediary metabolism of such tissues, particularly on the synthetic side.

A distinctive feature of the mammary gland is that it periodically undergoes changes of the most striking kind, both structurally and functionally. Thus, during the later stages of pregnancy, after structural development is complete, the gland is metabolically relatively quiescent; parturition, however, initiates a period of intense secretory activity which necessitates a considerable increase in energy exchange as evidenced by a prompt rise in respiration to a relatively high level. The transition from quiescence to activity is under hormonal control and thus mammary gland biochemistry is certain to become increasingly concerned with hormone and enzyme interactions, a topic which will soon be of outstanding importance in metabolic studies of many tissues.

There seems little doubt that the intermediary metabolism of the mammary gland is a promising field of study for the biochemist, a field which has been rather unproductive hitherto merely because relatively little effort has been expended upon it. So far, all we have at our disposal are general indications as to the nature of the blood precursors of the three major milk constituents. Even these basic findings are not yet beyond all doubt, while our knowledge of the mechanisms of the synthetic transformations is practically nil. The investigator working in this field will have at his disposal the immense amount of knowledge now accrued about enzyme-catalysed reactions in other tissues such as muscle, but since the mammary gland produces major products found nowhere else in nature, he must at the same time be prepared to seek new reactions in this tissue.

This article attempts a critical evaluation of the present position as it has developed since the topic was last reviewed in this journal (Folley, 1940). Aspects of the subject have subsequently been considered in reviews by Kay (1947), Petersen (1942, 1944) and Smith (1941, 1945). No attempt is made here to deal exhaustively with papers published earlier than 1941; they are only cited where relevant to the argument.

II. PROBLEMS OF TECHNIQUE

(1) *Arterio-venous technique*

So far the arterio-venous technique has been the most favoured one for metabolic studies of the mammary gland, doubtless because of all organs it is peculiarly suited for this purpose, being a more or less isolated organ, situated on the body surface and with afferent and efferent blood vessels which, in the case of ruminants, can be tapped with comparative ease (for technique see account by Folley, 1940). The principle of the method consists in taking samples of the blood entering and leaving the gland, as nearly simultaneously as possible, and comparing the concentrations of glandular metabolites or metabolic products in the two types of blood.

It was pointed out previously (Folley, 1940) that for quantitative interpretation of arterio-venous (A.-V.) differences an estimate of the ratio of mammary blood flow to rate of milk secretion (blood/milk ratio) is required. For instance, if one is testing the theory that lactose is formed from blood sugar it is desirable to determine whether a given blood sugar uptake (A.-V. difference) is sufficient to account for all the lactose secreted in a given time, which can only be done if the blood/milk ratio is known. The blood/milk ratio can be obtained (*a*) by direct measurements of the rate of blood flow through the udder or (*b*) indirectly by measuring the uptake of an element, such as calcium or phosphorus, which is absorbed by the gland in measurable amounts from the blood and the rate of secretion of which in the milk can readily be determined by appropriate analysis. In earlier days a practice had grown up of using A.-V. values determined for metabolites or suspected milk precursors such as sugar or fat for calculating blood/milk ratios (Lintzel, 1934; Graham Jnr., Jones & Kay, 1936; Shaw & Petersen, 1938*a*). Such estimates can legitimately be used to test quantitative aspects of a milk precursor hypothesis by comparing them with

independent estimates obtained as above, but to discuss them as estimates of the blood/milk ratio in their own right (as, for instance, was done by Pètersen, 1944 and Kay, 1945) involves an obvious logical fallacy. Recently, A.-V. differences in calcium and phosphorus have been used for estimating blood/milk ratios (see Table 1). The values indicate a ratio of 400–600/1 for the cow, a value about twice that obtained for the goat udder by Graham Jnr., Houchin, Peterson & Turner (1938) by direct measurement of blood flow with the thermostromuhr.

Table 1. *Blood flow-milk secretion ratios for ruminant udder*

Method	Animal	Blood volume / Milk volume	Reference
Thermostromuhr	Goat	150–250/1	Graham Jnr., Houchin, Peterson & Turner (1938)
Ca uptake	Cow	387/1	Shaw & Petersen (1938*a*)
Ca uptake	Cow	410/1*	Shaw & Petersen (1940)
Ca uptake	Cow	563/1†	Shaw, Powell Jnr. & Knodt (1942)
P uptake	Cow	425/1†	Shaw *et al.* (1942)
Ca+P uptake	Cow	494/1†	Shaw *et al.* (1942)

* Calculated on a blood plasma basis.
† Calculated on a whole blood basis.

There are certain points to bear in mind in considering these values. First, some of the calcium absorbed by the udder may be drained off in the lymph. Nothing quantitative is yet known about the lymph drainage from the udder, though Shaw & Petersen (1940) state that the flow of lymph from the udder is considerable and that the lymph contains appreciable amounts of calcium. The loss of some proportion of the calcium (and phosphorus) absorbed from the blood by the mammary gland by a route other than the milk would mean that blood/milk ratios estimated from A.-V. differences in calcium or phosphorus are underestimated to an unknown but probably significant extent. Secondly, it must be remembered that the A.-V. differences represent uptakes averaged over the relatively short periods required for collecting the blood samples and may thus for practical purposes be considered as 'instantaneous' values. The rate of secretion of the element in the milk, on the other hand, is usually obtained by analysis of the total milk secreted over a period of some hours, during which the rates of blood flow and of milk secretion may independently undergo considerable variation. Thus, if the blood samples are taken when the rate of calcium uptake happens (perhaps momentarily) to be relatively large, the blood/milk ratio will tend to be low, while the error will be in the opposite direction if the uptake at the time of sampling happens to be smaller than the mean. These, as it were, random fluctuations can of course be ironed out by taking the mean of a large number of determinations made under comparable conditions. More important may be systematic variations in uptake of certain milk constituents between one milking and the next. Thus Shaw & Petersen (1940) found that the uptake of calcium by the bovine udder was negligible immediately after milking and increased gradually, only becoming appreciable after some hours. The same applied to blood fat but not to sugar and amino-acids. These findings illustrate the probable

shortcomings of mammary gland fat balances even when computed, as by Shaw & Petersen (1940), on the basis of the mean of a number of A.-V. differences determined at various intervals after milking. The theoretically sound procedure, unfortunately hardly practicable, would involve determining and integrating curves for blood fat and calcium uptake between two milkings.

It is evident that the blood/milk ratios given in Table 1 cannot represent more than the merest approximation to the truth, and it is probable that the true mean ratios, in so far as average values have any useful meaning in the case of a quantity which must undergo considerable variation between one milking and the next, are likely to be greater than the published estimates.

Arterio-venous differences in mammary gland metabolites are also subject to uncertainties arising from the possibility of loss in the lymph (Graham, Jnr., Kay & McIntosh, 1936) and from random or systematic changes in the rate of uptake, a circumstance which increases the already almost insuperable difficulties in the way of conducting valid A.-V. balance experiments on the udder with the object of subjecting precursor hypotheses to the rigours of quantitative test.

In recent years an innovation has been proposed which, it is claimed, improves the technique of blood sampling, long recognized as one of the weak points of the A.-V. method. Shaw & Petersen (1939) observed that when the subject was noticeably disturbed during blood sampling, appreciable blood volume changes (we may assume probably due to vasomotor effects) occurred during the passage of the blood through the udder. These were usually manifested by A.-V. haemoglobin differences amounting to considerably more than 1%, and particularly affected A.-V. differences in non-diffusible metabolites such as blood fat or protein. Correction for the blood-volume changes, such as had previously been attempted by Graham Jnr., Peterson, Houchin & Turner (1938), gave, in these experiments, results which were clearly untenable. In order to minimize the risk of such vasomotor disturbances during blood sampling with their attendant errors, Reineke, Williamson & Turner (1941*a*) proposed the use of animals anaesthetized with Nembutal, an anaesthetic chosen because it does not alter the blood sugar level, changes in which, since glucose is an important mammary-gland metabolite, might affect milk secretion. These workers reported that goats under Nembutal anaesthesia continue at an unchanged rate to secrete milk of normal composition, and that the usual metabolites are absorbed by the udder under these conditions. Shaw (1946) has also reported unaltered milk secretion rate in cows anaesthetized with Nembutal.

Applying this technique, Reineke, Stonecipher & Turner (1941) claimed that mammary gland respiratory quotients (R.Q.) determined on anaesthetized goats were less variable than those obtained on unanaesthetized goats, or goats in which local anaesthetic had been applied at the site of venous puncture, provided anaesthesia was not excessive so that respiration was impaired. In the latter case values believed to be low were obtained. Shaw (1946), on the other hand, reported mammary R.Q.s for anaesthetized cows which are perhaps not outstandingly consistent, varying as they do from 0·78 to 2·11, though the mean A.-V. difference in Hb was only 0·48%.

While this latter value was not significantly lower than the mean A.-V. Hb difference obtained by Shaw & Petersen (1939) for non-excited non-anaesthetized cows, Shaw (1946) states that the individual values were more uniform.

In view of the considerations discussed above, it seems justifiable to conclude that quantitative balance experiments conducted on the mammary gland by the A.-V. technique have very little validity; the A.-V. technique in its present form is capable of giving qualitative results only.

(2) *Perfusion studies*

The isolated sheep's udder was perfused by Foà (1912) many years ago, but the perfusion technique has since hardly been used in this field until comparatively recently. A modern apparatus for perfusing the isolated bovine udder was described by Petersen, Shaw & Visscher (1941), who had already foreshadowed the final outcome in a brief publication (1939) in which they enumerated the advantages of this technique as applied to the mammary gland. The main advantages over the A.-V. method appear to be: (*a*) effects of general disturbance of the animal are eliminated; (*b*) the actual rate of blood flow through the gland at all stages of the experiment can be measured; (*c*) metabolites can be added to the perfusate and their fate studied; and (*d*) there is a possibility that the lymph can be sampled. Evidence that the isolated preparation functions normally, cited by Petersen, Shaw & Visscher (1939), was the disappearance of metabolites, particularly glucose, from the blood and the uniformity of R.Q. values. An obvious disadvantage of the perfusion method, mentioned by Reineke, Stonecipher & Turner (1941) in discussing R.Q.s less than unity obtained on perfused glands by some workers, is the difficulty of maintaining optimal concentrations of metabolites, hormones, etc., necessary for the normal functioning of the gland. Petersen *et al.* (1941) claim that the isolated, perfused udder can be maintained in good condition for some hours, so that modern technique evidently eliminates that rapid tissue deterioration which Meigs (1922) criticized so severely in respect of Foà's pioneer work.

Peeters & Massart (1947), unaware of the American perfusion work because of war conditions, have independently developed an apparatus, a novel feature of which is that it permits simultaneous but separate perfusion of each of the two halves of the bovine udder which is divided medially to eliminate venous anastomoses and thus prevent mixing of the venous blood from the two halves. The object is that the two halves (each consisting of two separate glands) can within reason be considered as comparable organs one of which may, if need be, serve as control for the other.

(3) *Tissue slices*

Slices of rodent mammae cut to a thinness according to Warburg's well-known directives were used by Grant (1935, 1936) in studies of lactose synthesis and recently by Folley & French (1948 *a–c*, 1949 *a–c*) for respiration studies. Tough connective tissue septa enclosing the lobules make the tissue difficult to cut, but the microtome of Stadie & Riggs (1944) is a great help. Large slices, stated to be 1–2 mm. thick,

cut from bovine udders with a commercial meat slicer, have been used by Knodt & Petersen (1946*a*) for intermediary metabolic studies.

One difficulty in work with mammary gland slices is that the tissue, consisting as it does of lobules of alveoli, tenaciously retains milk which cannot completely be removed by any known means. This may amount to anything up to 50% of the moist weight of the tissue (Folley & Greenbaum, 1947*a*). The difficulty of relating metabolic changes to tissue dry weight, for example for comparison with other tissues, is therefore obvious. It is not feasible to calculate results to a basis of protein-bound phosphorus as was done for various tissues by Rosenthal & Drabkin (1943), because milk contains casein which is a phosphoprotein. The milk content at the outset can be estimated from the tissue lactose content (Folley & Greenbaum, 1947*a*), but this cannot be applied to metabolic experiments because some milk escapes into the medium during incubation of the slices. The 'milk error' does not affect R.Q. determinations, but because of it Q_{O_2} values are probably underestimated. Another factor which may lead to underestimation of Q_{O_2} values is the unavoidable presence in the slices of varying amounts of connective tissue, presumably less metabolically active than the parenchyma, forming the septa which separate the lobules. The proportion of this may well vary from slice to slice cut from the same piece of tissue. The tissue also contains what is likely to be a fairly constant proportion of myoepithelium (see p. 329), the contribution of which to the total metabolism is unknown but may be equivalent to that of resting muscle.

(4) *Tracer isotopes*

A decade has passed since a tracer isotope was first used for studying the metabolism of the udder (Aten Jnr. & Hevesy, 1938), but since then only sporadic and not very informative applications of this technique have been made (see Kay, 1947). It may be anticipated that many hitherto intractable problems in this field, as already in so many others, will soon be solved by tracer studies.

III. RESPIRATION STUDIES

(1) *Oxygen uptake*

The first *in vitro* study of the respiration of mammary tissue appears to be that of Kleiber, Smith & Levy (1943) on the rat. Their results, obtained on 'portions' of mammary tissue which do not appear to have been slices in the accepted sense, indicate a progressive increase in $-Q_{O_2}$ from a value of 0·9 just before parturition to 2·9 on the twenty-first day of lactation. On the other hand, on a moist tissue basis there was no difference between the respiration rate on the twentieth day of pregnancy and on the twenty-first day of lactation, the explanation of the apparent discrepancy being that the dry-matter content of the gland during pregnancy is much higher than during lactation. It seems possible that the tissue in this study was not in equilibrium with oxygen since the respiration rates are much lower than those subsequently reported for the rat by Folley & French (1948*a*,*b* 1949*a*–*c*).

Studies with mammary gland slices in progress in this laboratory (Folley & French, 1948*a–c*, 1949*a–c*, and further unpublished work) indicate approximate $-Q_{O_2}$ (glucose) values at 37° for lactating mammary tissue of various species as follows: mouse, 16; rat, 10; guinea-pig, 8–9; rabbit, 6·5; goat, 5; cow, 3·5. Since, as we have seen (p. 321), these values must be to some extent underestimated, we may conclude that the lactating mammary epithelium respires fairly actively in comparison with other tissues. The most active gland studied, that of the mouse, bears comparison in this respect with rat liver and spleen, but is not as active as rat kidney. The only other sugar, besides glucose, found to be effective in raising the Q_{O_2} of rat tissue above endogenous values was mannose; glycogen, lactose, maltose, sucrose, galactose, fructose, glucosamine, arabinose and ribose were not utilized.

The initiation of copious lactation at parturition is accompanied by a striking increase in the respiration rate over a period of but 2 days; in the rat (Folley & French, 1949*c*), $-Q_{O_2}$ changes from approximately 1·3 on the twentieth day of pregnancy to approximately 4·4 on the first day of lactation. This rapid increase in respiratory activity must be under the ultimate control of complex hormonal events responsible for the initiation of lactation and would repay further analysis from this point of view. There is a further considerable increase in respiration between the first and the fifteenth day ($-Q_{O_2} = 10$) which seems to be correlated with the progressive increase in milk yield which, the work of Brody & Nisbet (1938) indicates, occurs during this period. In these studies the greater dry-matter content of pregnant gland compared with lactating gland, reported by Kleiber *et al.* (1943), has been confirmed, but, contrary to their findings, the respiration of lactating tissue has been shown to exceed that of pregnant tissue even on a moist tissue basis.

Oxygen uptake *in vivo* has been measured in numerous A.-V. studies on the ruminant udder made for the purpose of determining the R.Q. (for references see Table 2). An interesting feature is a tendency for the A.-V. differences in oxygen to remain more or less constant (approx. 3–5 vol. % in the cow) regardless of the functional condition of the gland. This is the case, for instance, for determinations by Reineke, Stonecipher & Turner (1941) on the udders of lactating and pregnant, non-lactating goats. Since the oxygen requirements of the gland are bound to increase during lactation, as the *in vitro* studies in fact show, it follows that the additional demands of lactation must be satisfied by an increase in the blood flow.

(2) *Glycolysis*

Mammary tissue (rat) shows appreciable anaerobic glycolysis which is considerably decreased in presence of oxygen (Folley & French, 1949*b*). There is thus a distinct Pasteur effect. It is known that mammary tissue produces lactic acid on incubation (Svanberg, 1930; Barrenscheen & Alders, 1932; Knodt & Petersen, 1945), but it seems possible that some of the still considerable aerobic acid production may consist of acids other than lactic, particularly when it is remembered that A.-V. studies provide no evidence that the lactating udder produces lactic acid *in vivo* (Powell Jnr. & Shaw, 1942).

Citric acid occurs in milk and, according to Knodt & Petersen (1946*a*), is produced on incubation of lactating udder slices alone or with various substrates (glycogen, maltose, glucose, lactate, pyruvate). It is thus possible that part of the aerobic acid production observed in rat mammary slices may be due to the formation of citric acid, perhaps as a by-product of the tricarboxylic acid cycle. Another possibility is that some of the aerobic acid production may be accounted for by free fatty acids synthesized by the gland. Graham Jnr., Houchin, Peterson & Turner (1938) believe that the gland can synthesize fat from carbohydrate (see p. 338), and Kelly & Petersen (1939) claim to have demonstrated the presence of free fatty acids in the mammary epithelial cells.

(3) *The respiratory quotient*

Arterio-venous studies of the respiratory exchange of the lactating ruminant udder have mostly given R.Q. values greater than unity (see Table 2). This has been

Table 2. *Determinations of mammary gland respiratory quotient*

Animal	Method	R.Q.	Reference
Non-lactating glands:			
Rat (20 days pregnant)	Tissue slice: glucose substrate	0·83	Folley & French (1949*c*)
Goat (pregnant)	A.-V.: Nembutal anaesthesia (?)	1·09	Reineke, Stonecipher & Turner (1941)
Goat (non-pregnant)	A.-V.: Nembutal anaesthesia (?)	0·81	Reineke, Stonecipher & Turner (1941)
Cow	A.-V.	0·76	Petersen & Shaw (1942)
Cow (non-pregnant)	A.-V.: Nembutal anaesthesia	0·61	Shaw (1946)
Cow	A.-V.: perfused, isolated udder	1·11	Petersen & Shaw (1942)
Lactating glands:			
Rat (15th day of lactation)	Tissue slice: glucose substrate	1·60	Folley & French (1949*b*, *c*)
Goat	Tissue slice: acetate substrate	1·20	Folley & French (1948*c*, 1949*a*)
Goat	Tissue slice: glucose substrate	0·86	Folley & French (1948*c*, 1949*a*, *b*)
Goat	A.-V.	1·36*	Graham Jnr., Houchin, Peterson & Turner (1938)
Goat	A.-V.: Nembutal anaesthesia	1·18*	Reineke, Stonecipher & Turner (1941)
Cow	A.-V.	1·25	Bottomley & Folley (unpublished)
Cow	A.-V.	1·20	Petersen & Shaw (1942)
Cow	A.-V.: Nembutal anaesthesia	1·27	Shaw (1946)
Cow	A.-V.: perfused, isolated udder	0·80	Shaw (1939)
Cow	A.-V.: perfused, isolated udder	1·05	Petersen & Shaw (1942)

* Corrected for carbon dioxide supposedly used for urea synthesis by the mammary gland.

interpreted (Graham Jnr., Houchin, Peterson & Turner, 1938; Reineke, Stonecipher & Turner, 1941) as indicating that some at least of the milk fat is formed in the gland from carbohydrate. The original results (on the goat) by Graham Jnr., Houchin, Peterson & Turner (1938) were rather variable as were those of Reineke, Stonecipher & Turner (1941) which were obtained on unanaesthetized goats, and excessive variability in respect of respiratory exchange measurements in the cow was commented on by Petersen & Shaw (1942). No undue variability, however, was encountered in a small series of determinations carried out in this laboratory on the

cow (Bottomley & Folley, unpublished) in which the blood samples were collected over mercury. Eight experiments in which no appreciable haemoconcentration occurred gave a mean R.Q. of $1{\cdot}25 \pm 0{\cdot}12$ (95% fiducial limits). Relatively uniform values were reported by Reineke, Stonecipher & Turner (1941) working with anaesthetized lactating goats; the crude mean value obtained by these workers, 1·09, could hardly be considered as significantly greater than unity but it was raised to 1·18 by use of a correction for carbon dioxide believed to be used for synthesis of urea by the mammary gland. The mean A.-V. urea difference was assumed to be the same as that previously observed by Graham Jnr., Houchin, Peterson & Turner (1938) and the same carbon dioxide correction factor was used.

When working with anaesthetized animals it is necessary to ensure that anaesthesia is not so deep that the respiration is impeded, otherwise false values may be obtained due to the lungs being unable efficiently to clear carbon dioxide from the blood and its consequent retention by the mammary gland. Reineke, Stonecipher & Turner report seven such cases which gave a mean R.Q. of 0·81. Shaw (1946) also used anaesthetized subjects (cows) but his values are perhaps not satisfactorily constant as we have seen (p. 319).

Respiratory quotient values determined on perfused lactating bovine glands were first reported as below unity (Shaw, 1939), but later a mean value of 1·05, somewhat higher but hardly different from unity, was reported by Petersen & Shaw (1942). Reineke, Stonecipher & Turner (1941), however, question the relevance to the intact gland *in vivo* of values obtained on the isolated perfused udder.

In vitro studies on mammary gland slices (glucose substrate) have amply confirmed the high R.Q. of the lactating gland for the rat, mouse, rabbit and guinea-pig (Folley & French, 1948*a–c*, 1949*a–c*, and further unpublished work). Values for the goat (eleven experiments) and cow (four experiments) all lay between 0·64 and 0·95 however, a curious anomaly which may indicate that the intermediary metabolism of the ruminant gland differs in important respects from that of non-ruminants. The probability that this is so, and the interest of these results, has been heightened by the further finding (Folley & French, 1948*c*, 1949*a*) that with acetate as substrate, mammary slices of the rat and mouse now give an R.Q. below unity, while tissue from the lactating goat gives a mean value of approximately 1·2. The possible significance of these findings is discussed on p. 342 *et seq.*

The most extensive *in vitro* studies have so far been made on the rat in which species it has been found that the R.Q. on the first day of lactation is nearly unity, but at all subsequent stages studied (8, 15 and 22 days) higher but fairly constant values, clustering round a mean of 1·6, have been obtained. Two days after weaning, however, the R.Q. has fallen to 0·75. The only other carbohydrate substrate so far found which will, like glucose, raise the low endogenous R.Q. (see below) is mannose (Folley & French, 1949*b*, *c*).

Respiratory quotients mainly below unity have been reported for non-lactating ruminant udders (see Table 2) and also for glands of fasted lactating ruminants (Reineke, Stonecipher & Turner, 1941; Shaw, 1946). The latter may not unreasonably

be considered as analogous to lactating mammary slices incubated without substrate, for which values between 0·7 and 0·8 (rat) were obtained at all stages of lactation (Folley & French, 1949*b*, *c*), possibly indicating that fat is oxidized by the slice under these conditions. Even partial fasting tends to lower the mammary gland R.Q. Slices from lactating rats on a restricted food intake for 13 days (as also from adrenalectomized lactating rats with which the former were pair-fed) gave R.Q.s (glucose) of approximately unity, i.e. intermediate between the endogenous and 'glucose' values for slices from normal rats in full lactation. Adrenalectomized and pair-fed rats alike were lactating when killed, but at a considerably reduced level (Cowie, Folley, French & Greenbaum, 1949; Folley & French, 1949*b*). In agreement with the bulk of the determinations on ruminants by the A.-V. method (Table 2) the mammary R.Q. (glucose) was low (0·83) in the rat at the twentieth day of pregnancy, the contrast with post-partum values providing another indication of the hormone-induced change in the metabolism of the mammae at term (Folley & French, 1949*b*).

The respiratory quotient even of a single organ, no less than of the whole animal, is often difficult to interpret since it can only represent the overall gas exchange due to numerous reactions (see Soskin, 1941). Whether the high R.Q. which undoubtedly seems to be characteristic of the lactating mammary gland, at any rate in most species, really does mean that a considerable proportion of the milk fat (perhaps in the case of ruminants the lower fatty acids in particular, as suggested by Reineke, Stonecipher & Turner, 1941), is synthesized from carbohydrate precursors, perhaps needs further investigation. The *in vitro* results of Folley & French (1948*c*, 1949*a*) would seem to suggest that in the case of ruminants, the high udder R.Q. is indicative rather of fat synthesis from acetate (see p. 342). In any event there seems little doubt that mammary tissue, of ruminant and non-ruminant alike, is noteworthy in its ability to effect fat synthesis from a single substrate *in vitro* and would appear to offer attractive possibilities for fat synthesis studies.

(4) *Source of energy of the normal lactating gland*

The nature of the substrates from which the gland derives its energy is still largely obscure. Arterio-venous studies have uniformly shown an uptake of glucose by the lactating ruminant udder, often of the order of 10–15 mg. %, which for most tissues would suffice to point to glucose as the principal fuel. Indeed, Graham Jnr., Houchin, Peterson & Turner (1938) suggested that the high R.Q. of the lactating ruminant udder is incompatible with the oxidation of anything but carbohydrate. However, the mammary gland is exceptional in that it synthesizes large amounts of a disaccharide, lactose, the chief if not the sole precursor of which, according to the balance of evidence so far obtained, must be glucose. Balance studies on the udder by the A.-V. method (Graham Jnr., 1937; Shaw, Boyd & Petersen, 1938; Shaw & Petersen, 1938*a*; Shaw, Powell Jnr. & Knodt, 1942) indicate that if glucose is the precursor of lactose then there can be little if any left over for oxidation. It is doubtful whether the discovery by Reineke, Williamson & Turner (1941*b*) of an additional uptake of carbohydrate in the form of glycoprotein would do much to

ease the dilemma of those (Graham Jnr., Houchin, Peterson & Turner, 1938; Reineke, Stonecipher & Turner, 1941) who, on the basis of the high R.Q., find an additional destiny for carbohydrate in the mammary gland, namely, for synthesis of fat. However, the apparent dilemma may ultimately prove not intractable in view of the aforementioned uncertainties associated with balance experiments by the A.-V. technique and also because it is now gradually being realized that in ruminants, on which all A.-V. work has been done, hitherto unsuspected metabolites, e.g. acetate, arising from fermentation in the rumen, are available to the udder for the synthesis of fat and perhaps other major milk constituents as well as for oxidation. In fact the studies of Folley & French (1948*c*, 1949*a*, and further unpublished work) on the oxidation of acetate by mammary gland slices, backed by McClymont's (1948) demonstration of an A.-V. difference in acetate for the bovine udder (see p. 342) leave little doubt that acetate serves as a source of energy for the mammary gland, at least in the ruminant.

The idea that a proportion of the fatty acids arising from blood glycerides absorbed by the mammary gland is partially oxidized was put forward by Shaw & Petersen (1938*a*, 1940) since in balance studies by the A.-V. method they found a greater uptake of blood fat than was necessary to provide the milk fat formed. In later experiments, however, Shaw *et al.* (1942) observed an uptake of blood fat sufficient to account only for about 93% of the milk fat. Nevertheless, as a result of a study of the ketone body metabolism of the bovine udder, Shaw (1942) believed that about 63% of the oxygen used by the gland was utilized for oxidation of fatty acids other than β-hydroxybutyric acid. An appreciable uptake of this latter intermediate in fat metabolism by the lactating bovine mammary gland had been observed by Shaw & Knodt (1941*a*) who considered two possibilities: (*a*) that it serves as a precursor of the short-chain fatty acids of milk fat, and (*b*) that the gland derives energy by its oxidation. Since the percentage of short-chain acids in milk fat decreases in ketosis when the blood ketones are high (Shaw, 1941*b*) while the uptake of β-hydroxybutyric acid increases under these conditions (Shaw, 1942), Shaw's earlier inclination (Shaw & Knodt, 1941*a*, *b*) to favour the first possibility was reversed in favour of the second (see also Shaw *et al.* 1942). His results (Shaw, 1942) indicate that if this substance is indeed oxidized, about 37% of the total oxygen consumed by the udder is required in the normal cow, while in ketosis the enhanced uptake would require most or all of the oxygen. Ketone balance studies on the perfused isolated bovine udder in which high blood sugar levels were maintained (Shaw & Petersen, 1943) contra-indicate the possibility that in ketonaemia the gland absorbs, and presumably oxidizes, more β-hydroxybutyric acid than normally, merely because the blood sugar is lowered. Moreover, since the glucose uptake seems to be undiminished in ketosis (Shaw, 1941*a*) it appears improbable that the gland of the normal animal oxidizes glucose in preference to β-hydroxybutyric acid. Shaw (1942) reported a mean R.Q. of 1·15 for the udder of the cow in ketosis but does not consider this valid evidence against the theory that in this condition the whole of the oxygen utilized is used for the oxidation of β-hydroxybutyrate (theoretical R.Q. = 0·89)

IV. MAMMARY GLAND ENZYMES

(1) *Enzymes present*

A number of enzymes have been found to occur in lactating mammary tissue; of these only the alkaline phosphatase and arginase have been extensively studied. Since the subject was last reviewed in this journal (Folley, 1940) a number of additional enzymes have been identified in this tissue. These comprise an enzyme, desoxyribonuclease, said to cause depolymerization of thymonucleate (Greenstein & Jenrette, 1941; Greenstein, 1942*a*), catalase and xanthine dehydrogenase (Greenstein, Jenrette, Mider & Andervont, 1941) both of which are known to occur in milk, and acid phosphatase (Greenstein, 1942*b*; Dempsey, Bunting & Wislocki, 1947). This last is presumably the phosphomonoesterase A_2 in the phosphatase classification of Folley & Kay (1936). Cytochrome *C* has been determined in the mammary tissue of the pregnant rat (Rosenthal & Drabkin, 1943) indicating the probable presence of the hydrogen transport enzymes which collaborate with cytochrome.

(2) *Alkaline phosphatase*

A method for the purification of the mammary gland alkaline phosphatase has been described by Caputto & Marsal (1941). Purified preparations (bovine) so obtained (Caputto & Marsal, 1944*a*) contain about 10% nitrogen of which about one-twentieth to one-twelfth is free amino nitrogen. The molecule contains organically bound phosphorus which is considered to be related to the catalytic activity since three preparations showed a fairly constant activity/phosphorus ratio. The enzyme was resistant to the action of trypsin and its properties were not those of a globulin since it was not precipitated by half saturation with ammonium sulphate. With increasing purification the ultra-violet absorption spectrum approached more and more that of a protein rich in aromatic amino-acids (Caputto & Marsal, 1944*b*).

Folley & Kay (1935), as a result of a thorough comparison of the properties of the alkaline phosphatase, later (Folley & Kay, 1936) classified as a phosphomonoesterase A_1, present in crude extract of guinea-pig mamma with those of similar kidney preparations, concluded that the two enzymes are identical. Caputto & Marsal (1944*a*), however, on the basis of studies of the action of various inhibitors on their purified preparation, believe that it differs from the 'alkaline' enzyme of kidney and liver, but is identical with the enzyme present in blood plasma, intestinal mucosa and bone. Subdivision along these lines of the phosphomonoesterases belonging to the class A_1 of Folley & Kay (1936) on the basis of behaviour towards activators and inhibitors had previously been suggested (e.g. by Cloetens, 1939), and Caputto & Marsal (1944*a*) evidently accept such criteria as adequate for establishing the non-identity or otherwise of alkaline phosphatases present in different tissues. It is, however, questionable whether the properties of purified enzyme preparations or of enzymes present in crude tissue extracts or homogenates are the more representative of those of the enzymes in their 'native' state. In point of fact there is, as suggested by Folley & Greenbaum (1948*a*) in the case of arginase, some reason to believe that

the properties of purified enzymes may, in some cases, be misleading in this regard. This is illustrated in the case of the alkaline phosphatase by the work of Thoai, Roche & Roger (1947) which indicates that on purification the enzyme loses considerable specificity in its requirements respecting activation by divalent metal ions. In any event further evidence would be desirable before the non-identity of the alkaline phosphatases of kidney and mammary gland can be taken as established.

Changes in the concentration of alkaline phosphatase in the rat mammary gland during pregnancy, lactation and involution (Folley & Greenbaum, 1947*a*) are shown in Fig. 1. The increase in the enzyme content of the gland, beginning at about the

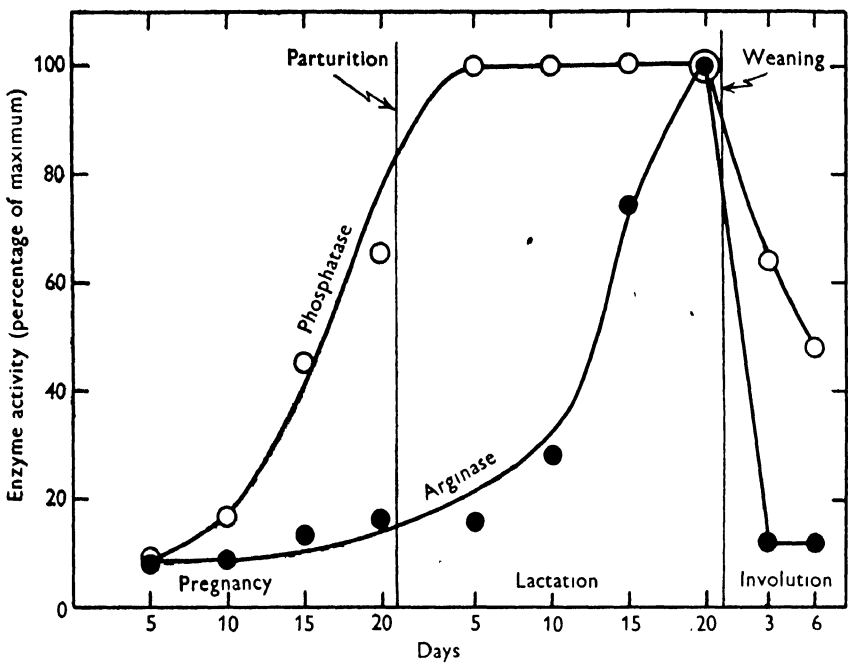

Fig. 1. Changes in the arginase and alkaline phosphatase content of the mammary gland of the rat in pregnancy, lactation and involution. The data, expressed on a moist tissue basis corrected for retained milk, are taken from Folley & Greenbaum (1947*a*).

middle of pregnancy, is quite striking and the final high level (about twelve times that of the beginning of pregnancy) is maintained constant throughout lactation. These changes unfortunately throw very little light on the function of the alkaline phosphatase in lactating mammary gland. It is, however, perhaps permissible to conclude that the enzyme is concerned with the function of the gland rather than with its growth since the increase in enzyme concentration is virtually confined to the latter half of pregnancy, by which time the development of the gland is almost complete and the secretory phase has begun (see Folley & Greenbaum, 1947*a*). The possibility that the mammary enzyme is concerned with the capture and absorption of sugar molecules from the blood, as suggested by Moog (1946) in respect of the kidney enzyme, would be attractive were it not for the unlikelihood of the absorbed

hexose phosphate undergoing dephosphorylation prior to its further utilization by the mammary gland. Another possibility, which though extremely vague and speculative should not be overlooked, is that the enzyme may be concerned with synthesis of milk protein. It has been pointed out (see Moog, 1946) that phosphatases are often found in cells in which active protein synthesis is proceeding and that in such cells there is frequently a correlation between content of pentose nucleic acid, thought to be an instrument of protein synthesis, and of phosphatase.

Species differences in the concentration of alkaline phosphatase in the lactating gland have not yet been studied, nor have we adequate information about the activity of the gland in relation to other tissues. In the rat, Folley & Greenbaum (1947*a*) found the phosphatase activity of the lactating mammary gland to be about one-third that of the kidney.

The localization of alkaline phosphatase in the mammary gland has been studied by Dempsey *et al.* (1947) using a histochemical method. It will be recalled that the ducts of the resting mammary gland are lined by a double layer of epithelial cells which proliferate during pregnancy to form the extensive inter-and intra-lobular duct system and the alveoli. The single layer of secretory epithelium lining the alveoli and the majority of the finer ducts is regarded by most histologists as the product of the inner epithelium of the resting ducts, while the cells in the outer layer of these ducts become transformed into extremely elongated myoepithelial cells interposed between the secretory epithelium and basement membrane. There has been some uncertainty in the past regarding the identity, orientation, quantitative distribution and function of the so-called myoepithelial or basket cells, and some authors have added to the confusion by calling the outer epithelium of the resting ducts myoepithelium although these cuboidal cells are really only potential myoepithelium. Others have abandoned the term altogether in favour of smooth muscle (see Richardson, 1949). Dempsey *et al.* (1947), using the Gomori technique for histochemical demonstration of alkaline phosphatase, found in the non-pregnant rat a high phosphatase content in the outer epithelium of the immature ducts (which they termed myoepithelium) and only a moderate staining of the inner epithelium. The surrounding stroma and capillary plexuses were phosphatase-negative. The formation of alveoli, true myoepithelium and a capillary plexus in close contact with the alveolar walls, typical of the thirteenth day of pregnancy, was accompanied by a redistribution of the enzyme so that it was now concentrated in the myoepithelial zone and in the capillary walls. Towards the end of pregnancy and during lactation the enzyme appeared to be localized almost entirely in the capillary endothelium. Dempsey *et al.* (1947) interpret their findings as indicating the presence of a barrier of enzyme between the blood supply and the mammary epithelial cells which, drawing an analogy with what is believed might be the role of phosphatase in other tissues such as the intestinal epithelium, the placental syncytium, etc. (see Moog, 1946), they suggest subserves a function connected with the transport of metabolites. This study would seem to assign a secondary role among mammary structures to the alveolar epithelium as a site of phosphatase activity, though the authors refer to

species differences in this respect and state that while in the rat the activity declines until none can be detected in alveolar epithelium during lactation, alveolar activity is relatively intense in the cat. Alkaline phosphatase has also been detected histochemically in the epithelium of the human mammary gland by Kabat & Furth (1941). However, granted that the application of the fashionable histochemical test for tissue phosphatase to tissues such as the mammary gland is likely to provide information which classical methods of phosphatase assay are unable to give, it seems doubtful whether the technique is capable of doing much more than indicating the presence or (with less certainty) absence of the enzyme; quantitative assessment of comparative potencies would seem to be outside its present scope. That being so, and in view of the technical difficulty of adequately differentiating myoepithelial cells in the lactating gland (see Richardson, 1949), which raises the question whether the alcohol or acetone fixation inseparable from the histoenzymatic test is adequate for the purpose, it is perhaps safest for the present to treat the above-mentioned claims respecting the distribution of alkaline phosphatase among the mammary tissues with reserve.

However, the problem of the formation of mixed tumours in the human and canine mamma might be pertinent in the present connexion. Such authors as Peyron, Corsy & Surmont (1926) and Biggs (1947) have claimed that the differentiation of mesenchymal components, such as bone, in these tumours can be traced to an initial hyperplasia of myoepithelium derived from the outer epithelium of the mammary ducts. Although many pathologists are unwilling to accept the theory that bone can arise from the activity of cells of epithelial origin, the fact that myoepithelial cells or their precursors are said to contain such an unusually high concentration of alkaline phosphatase demands further investigation of this much debated field.

(3) *Arginase*

The presence of arginase in mammary tissue, first reported by Shaw & Petersen (1938*b*), working on the bovine udder, has since been confirmed in the mouse (Greenstein, Jenrette, Mider & White, 1941) and rat (Folley & Greenbaum, 1946, 1947*a*).

The properties of rat mammary gland arginase as it occurs in tissue homogenates have been extensively studied by Folley & Greenbaum (1948*a*). The optimum pH of the enzyme is pH 9·45 and it obeys the Michaelis-Menten law at this pH but not at more acid pH at which excessive substrate concentrations cause inhibition. The 'native' enzyme is believed to be a manganese-protein which is easily, but reversibly, dissociated, for example by mere dilution. Extraction of the enzyme from the tissue inevitably involves partial inactivation due to dissociation of the manganese-protein and while reactivation can be achieved by addition of Mn^{++}, Co^{++} is much less effective despite the prevalent belief that it is an arginase activator. The properties of the enzyme present in liver homogenates are so similar that there is no reason to doubt that the mammary gland and liver enzymes are identical.

Changes in the arginase content of the rat mammary gland in pregnancy, lactation

and involution (Folley & Greenbaum, 1947*a*) are shown in Fig. 1. The rapid increase in the arginase concentration which begins at about the fifth day of lactation and continues to a peak at the twentieth day is noteworthy, as is the fact that the curves for arginase and phosphatase are quite dissimilar. Following weaning the arginase level falls back much more rapidly than does the phosphatase. The changes in the mammary arginase levels during lactation are discussed in relation to the probable function of the enzyme in the lactating mammary gland in certain species in another section (p. 344).

Folley & Greenbaum (unpublished work) have determined the arginase content (corrected approximately for the milk retained in the tissue) of the lactating mammary gland in various species (Table 3). It is seen that the enzyme content of the mammary

Table 3. *Mammary gland arginase levels in various species*
(Folley & Greenbaum, unpublished)

Animal	Mammary gland arginase units*/g. moist tissue	
	Absolute	Relative
Rat (17 days lactation)	145	100
Mouse (15 days lactation)	89	61
Mouse (5 days lactation)	51	35
Guinea-pig (10 days lactation)	10	7
Guinea-pig (5 days lactation)	7·8	5
Rabbit (28 days lactation)	4·0	3
Goat (lactating glands: stages of lactation unknown)	3·8	3
Cow (lactating glands: 71 and 208 days lactation)	3·5	2
Rabbit (7 days lactation)	1·9	1

* Folley & Greenbaum, 1948*a*.

gland in ruminants and other herbivores is low by comparison with the rat and mouse—in fact in the rabbit in early lactation hardly any enzyme could be detected by the sensitive method used. In view of the considerable changes in mammary arginase content which occur during lactation in the rat (Fig. 1), it would be preferable, in making species comparisons, to study the curve for each over the whole lactation period so that the maximum values can be compared. Since this formidable task could not be undertaken, the relative activities given in Table 1 are only approximate. Nevertheless, it seems fairly certain that the dictum of Folley & Greenbaum (1946, 1948*b*) to the effect that the mammary gland is the second most abundant source of mammalian arginase does not apply to the rabbit, nor probably to other herbivores, though it is well established for muridae and would probably hold for carnivores. In the rat in full lactation (seventeenth day) the mammary gland exhibits about one-ninth of the arginase activity of the liver (Folley & Greenbaum, 1948*b*), itself appreciably higher than during pregnancy (Folley & Greenbaum, 1947*a*).

V. CARBOHYDRATE METABOLISM

(1) *Precursors of lactose*

Work carried out up to 1940 (see reviews by Folley, 1940 and Petersen, 1944) had led to the conclusion that glucose must be the principal precursor of lactose with the possibility that a proportion of the latter may arise from other blood constituents such as lactate and amino-acids. More recent A.-V. studies (Powell Jnr. & Shaw, 1942; Shaw, 1946) indicate no uptake of lactic acid by the lactating bovine udder provided blood sampling causes no upset to the animal; it is implied that positive A.-V. differences previously observed (Graham Jnr., 1937; Shaw *et al.* 1938) must have been the result of manipulative disturbance leading to a rise in the arterial blood lactate with temporary retention by the mammary tissues. It would thus appear that blood lactate must be eliminated from consideration as a lactose precursor. Nor does there appear to be any uptake of pyruvate from the blood by the lactating cow udder (Shaw, 1946).

It has already been indicated that quantitative aspects of blood sugar utilization by the mammary gland are beset with paradoxes. While mammary gland carbohydrate balances, for what they are worth as evidence, disagree as to the adequacy of the glucose uptake to account for the observed lactose secretion, they are unanimous in indicating that, supposing glucose is the precursor of lactose, there can be little if any to spare for other purposes—oxidation, formation of fat—for which evidence exists. Additional sources of carbohydrate, or other substrates which may be utilized for these purposes, must therefore be sought. One such possible source is indicated by the observation of Reineke, Williamson & Turner (1941*b*) of a positive A.-V. difference in glycoprotein, for the udder of the lactating goat, corresponding to an uptake of about 2·15 mg. protein-bound carbohydrate (calculated as galactose-mannose) per 100 ml. blood. Further lactose precursors may arise by gluconeogenesis from amino-acids absorbed as such from the blood, for which absorption there is a certain amount of evidence (see p. 343 below), or arising from protein, for example the glycoprotein fraction just discussed. The possibility that the lactating mammary gland, at any rate in some species, is capable of effecting gluconeogenesis from protein is fully discussed in a later section (p. 343 *et seq.*).

The *in vitro* synthesis of lactose from glucose by lactating mammary gland slices has been generally accepted on the basis of experiments on guinea-pig tissue by Grant (1935, 1936) who used specific yeasts for the determination of lactose and galactose. Knodt & Petersen (1945) have since reported *in vitro* lactose synthesis by cow udder slices from glucose, maltose and glycogen. It must be conceded, however, that there is an element of doubt about the outcome of all these experiments because of the lack of a really satisfactory and specific chemical method for the determination of lactose in the presence of other sugars (a difficulty which we may hope will soon be overcome), though the methods used by Grant would seem to be less open to criticism than those used by Knodt & Petersen. Indeed, recent experiments by Malpress & Morrison (1948), in which a more specific method for lactose

determination is being used, indicate that to infer lactose synthesis *in vitro* from an increase in non-fermentable reducing power may be wrong. In the opinion of these workers reports published hitherto (except Grant, 1935, 1936) have not demonstrated such a synthesis by *in vitro* techniques. Knodt & Petersen (1945) themselves were apparently not unaware of a degree of uncertainty attaching to their results since they speak of 'the determination of a fraction which is referred to as lactose'. Full details of the conditions of their experiments are not given, so in particular we do not know whether the slices were adequately oxygenated. They were stated to have been on the average 2 mm. thick in the earlier stages of the work and though the average thickness was later reduced to 1 mm. it is probable that they were still too thick for proper penetration of oxygen (and perhaps of substrate too) even if the oxygenation of the medium was adequate. Thus an important point still remains obscure, whether mammary gland slices are capable of synthesizing lactose anaerobically at the same rate as in presence of oxygen if at all.

Table 4. *Utilization of various carbohydrates by the lactating mammary gland*

Substrate	Lactose formation	Oxidation‡	Presumed fat formation‡ (high R.Q.)
Glucose	+*†	+	+
Fructose	−*	−	−
Mannose	−*	+	+
Galactose	−*	−	−
Maltose	+†	−	−
Glycogen	+†	−	−

Compiled from the data of Grant (1935) on the guinea-pig*, Knodt & Petersen (1945) on the cow† and Folley & French (1949*b*) on the rat‡.

Sugars apparently utilized for lactose synthesis by mammary gland slices are shown in Table 4, together with those that raise the Q_{O_2} or are used for the presumed formation of fat as evidenced by a high R.Q. This table is compiled from the results of Grant (1935) for the guinea-pig, Knodt & Petersen (1945) for the cow and Folley & French (1949*b*) for the rat. Because of differences between species it is not yet possible to draw any conclusions about the relation of lactose synthesis to oxidation or fat synthesis. Thus if results with goat udder were included in the table a negative would have to be entered in the fourth column against glucose since although glucose slightly increases both the Q_{O_2} and R.Q., the latter is still below unity. Further work on *in vitro* lactose formation in various species is urgently needed.

(2) *The role of glycogen*

Mammary tissue contains glycogen but there is disagreement about how much. Barrenscheen & Alders (1932) reported results for mammary tissue from four guinea-pigs, two of which were lactating, corresponding to 30–40 mg. %, levels which they considered as negligible and inconsistent with any role for glycogen in the carbohydrate metabolism of the gland. On the other hand, Petersen & Shaw (1938) have

given 200 mg. % as a mean figure for cow udder tissue, dry or lactating, a magnitude sufficient to indicate a role for glycogen in the carbohydrate metabolism of the gland, particularly when it is remembered that correction for the milk content of lactating tissue (Folley & Greenbaum, 1947*a*) might almost double the value. Mammary glycogen levels more in accord with those of Barrenscheen & Alders than of Petersen & Shaw have been recently found by Folley & French (unpublished) as follows: rat (twenty-first day of lactation) 22 mg. %; goat (lactating and dry glands) 14–51 mg. %; though somewhat higher values (52–141 mg. %) were found for a series of cow udders, some containing milk, some dry. Glycogen levels of mammary tissue can be experimentally increased. Knodt & Petersen (1945) found that low glycogen levels of *c.* 25 mg. % characteristic of cow udders brought from the abattoir and stated to be low because of post-mortem glycogenolysis, could be raised to levels of about 200 mg. % by perfusion of the glands with blood containing high concentrations of glucose. When insulin was added to the perfusate (Knodt & Petersen, 1946*b*) the glycogen concentration was increased even more. In the rat, Folley & French (unpublished) found that the glycogen content of the mammary gland could be increased, in some cases considerably, by removal of the young some time before autopsy. Increased glycogen levels were found up to 3 or 4 days after experimental weaning.

Knodt & Petersen (1945) have considered possible roles for glycogen in the biological synthesis of lactose. They reported that incubation of cow udder tissue which had been enriched in glycogen by perfusion with hyperglycaemic blood caused the breakdown of glycogen and the appearance of lactose. This suggested two alternatives, either that glycogen is an obligatory intermediate in lactose formation or that the polysaccharide forms a reserve of carbohydrate which furnishes lactose precursors when the need arises. Later experiments (Knodt & Petersen, 1946*b*) in which insulin was added to the perfusion fluid were considered by these workers to favour the first alternative since milk secreted by insulinized perfused udders contained less lactose than that secreted by control udders. The deficit in lactose could not be accounted for by the increased glycogen content of the tissue.

The increase in mammary gland glycogen resulting from experimental weaning would fit in with either alternative. If milk is not removed from the gland lactose synthesis will slow down and finally stop. This might well cause glycogen, if it were an intermediate, to accumulate to a certain extent; on the other hand, if glycogen were merely a carbohydrate reserve it would still tend to increase under these conditions since glucose which otherwise would be utilized for lactose formation would be available for glycogen formation.

The distribution of glycogen among the different tissues of the mammary gland might well repay study by histochemical methods. The myoepithelium would be of particular interest in this connexion since investigators of the physiology of the suckling or milking process suggest (see Folley, 1947) that the expulsion of milk from the alveoli involves a neurohormonal reflex evoking contraction of a hitherto unidentified tissue which Richardson (1949) has recently shown with virtual certainty

to be the myoepithelium. If this is so, it would seem likely that the myoepithelial cells can store glycogen in periods of rest between successive milkings and it may be that a considerable proportion of the mammary gland glycogen is present in these contractile cells, which according to Richardson (1949) comprise a far from negligible element in comparison with the epithelium.

(3) *Possible mechanisms of lactose synthesis*

The synthesis of lactose by the mammary gland is essentially a problem of biological disaccharide synthesis, a field in which considerable advances are being made at the present time. This field, principally as it relates to the formation of sucrose in plants, has been the subject of an interesting review by Doudoroff (1945). Virtually nothing is known of the intermediate stages of lactose synthesis at the present time, for though *in vitro* synthesis has been effected by the use of tissue slices as we have seen, no one has yet succeeded in effecting unequivocal lactose formation by cell-free tissue extracts (see Folley, 1940).

The origin of the galactose moiety of the lactose molecule has so far defied all efforts to account for it. The conversion of glucose to its isomer galactose is a difficult task even in the chemical laboratory, for it has only been reported once (Oldham & Robertson, 1935). There seem to be three possibilities: (*a*) galactose may be formed as such in the mammary gland itself and then condensed with glucose, (*b*) galactose may not even momentarily exist in the free state but the necessary inversion of configuration at C_4 may occur at, say, the disaccharide stage, and (*c*) the galactose may be formed elsewhere in the body, as its existence as a constituent of cerebrosides, the carbohydrate moiety of serum glycoproteins, etc., shows that it can be, and transported in the blood to the mammary gland.

The third possibility would seem to be ruled out of serious consideration because (*a*) mammary slices synthesize lactose *in vitro* from glucose alone, and (*b*) isolated perfused udder preparations appear to continue synthesizing lactose so long as the glucose content of the perfusate is replenished. The amount of galactose originally present in the perfusion blood, say in the carbohydrate moiety of the serum protein, would hardly be sufficient to provide the galactose moiety of the lactose synthesized during the perfusion. Both these considerations indicate that the mammary gland needs no outside source of galactose and is capable of effecting the required configurational inversion itself. Moreover, Grant (1936) obtained no more synthesis of lactose by guinea-pig mammary gland slices in the presence of mixtures of glucose and galactose than would have been expected from the glucose alone. He interpreted these results as evidence against the two-stage synthesis:

$$\alpha\text{-glucose} \rightleftharpoons \beta\text{-galactose},$$

$$\alpha\text{-glucose} + \beta\text{-galactose} \overset{\beta\text{-galactosidase}}{\rightleftharpoons} \text{lactose},$$

which is essentially the first possibility enumerated above. This, however, as will be seen below, does not rule out the possibility that not galactose itself, but a galactose phosphate may first be formed and condensed with glucose. Galactose then would

not increase the lactose formation from glucose by tissue slices unless the mammary gland contains the galactokinase recently described by Trucco, Caputto, Leloir & Mittelman (1948) and so could phosphorylate this sugar. The effect of β-galactose-1-phosphate on lactose synthesis by mammary slices in presence of glucose has not yet been tested, but even if this were done with negative results, as in the case of galactose-6-phosphate (Grant, 1936), the outcome would not be decisive since the galactose phosphate might not be able to penetrate the epithelial cells. The relative impermeability of mammary gland cells to hexose phosphates was indicated by the fact that Folley & French (1949*b*) found glucose-1-phosphate to have no effect on the Q_{O_2} and R.Q. of rat mammary slices despite the high concentration of phosphatase, which presumably would split the ester if it penetrated the cells, in the rat mammary gland (Folley & Greenbaum, 1947*a*).

A possible route from glucose to galactose-1-phosphate has perhaps been opened up by the claim of Trucco *et al.* (1948) to have effected the enzymatic conversion of galactose-1-phosphate to glucose-6-phosphate. If this claim which has only as yet been stated in preliminary form is confirmed and, what is most important for our present speculations, all stages are shown to be reversible, then the possibility of postulating a feasible scheme for lactose synthesis, which should prove very stimulating for further investigation, is immediately apparent. Such a scheme, additionally postulating the presence in the mammary gland of a lactose phosphorylase capable of catalysing a reaction analogous to the reversible phosphorolysis of sucrose by *Pseudomonas saccharophila* described by Hassid, Doudoroff & Barker (1944), and involving, except for the hypothetical reactions just considered, in other respects known reactions, is given below (Fig. 2).

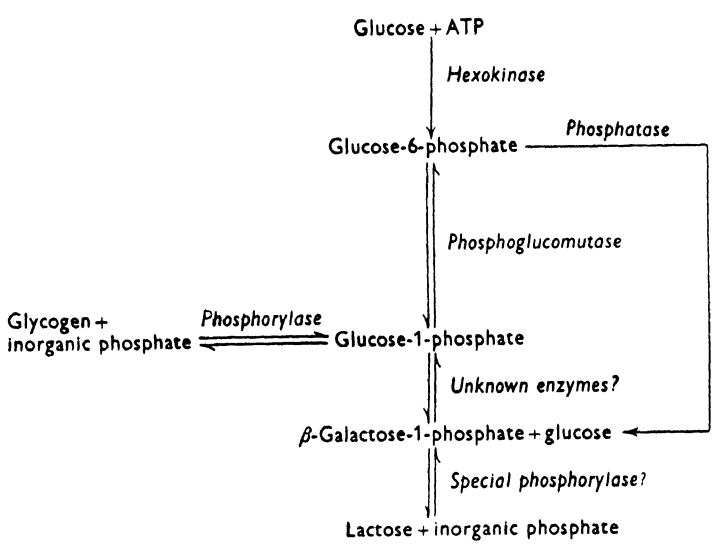

Fig. 2.

Two points may be noted about this scheme. First, it requires an external source of energy such as provided by respiration or glycolysis; and secondly, it casts

glycogen in the role of a carbohydrate reserve and not as an obligatory intermediate. We do not yet know whether lactose formation is linked with respiration but the results of Grant (1936), who observed inhibition of lactose synthesis by mammary slices in presence of fluoride or iodoacetate indicate, but as Grant pointed out not decisively, that it is dependent on glycolysis.

Doudoroff (1945), in considering possible mechanisms for sucrose formation, was led to consider mechanisms alternative to a condensation of glucose-1-phosphate with fructose because of the difficulty of reconciling the equilibrium constant of this reversible reaction with the high concentration of sucrose found in plants. On such thermodynamic grounds he pointed out the possibility, even probability, that the synthesis involves an irreversible process, such as hydrolysis of a phosphate ester, as the final step. In the case of the scheme proposed above for the synthesis of lactose, it is of course possible that the equilibrium constant might be such as to favour synthesis. If not, and if the passage of lactose from the site of synthesis in the mammary epithelial cell into the alveolar lumen does not take place under conditions such that equilibrium is displaced in the direction of synthesis, it is not impossible that the condensation might involve phosphates of both glucose and galactose leading to the formation of a lactose phosphate which would then irreversibly be removed from the reaction sequence by the removal of the ester phosphate under the influence of the alkaline phosphomonoesterase abundantly present in the mammary cells. Such an irreversible dephosphorylation would have the advantage of providing for the first time a clear cut and intelligible role for the mammary gland phosphatase. An analogous possibility was discussed by Doudoroff (1945) in relation to the formation of sucrose in higher plants.

VI. FAT METABOLISM

(1) *Precursors of milk fat: general considerations*

It is now generally agreed that the earlier accepted view which held that milk fat arises from blood phospholipins is untenable. This conclusion, based on A.-V. studies (see review by Folley, 1940), is entirely within the competence of the technique since only a qualitative decision is involved—is there or is there not a measurable A.-V. difference in phospholipin?—and the negative has been unanimous. These same studies and others more recent have focused attention on the neutral blood lipins, particularly the glyceride fraction, as an alternative possibility for the precursors of milk fat, but it must be remembered that the analytical methods used in most of these investigations have been such that other fractions of the neutral blood lipins (e.g. cholesteryl esters and, in the case of the method of Allen, 1938 which has been used in many of the later studies, cholesterol and fat-soluble pigments) are estimated along with the triglycerides. Therefore the possibility that fatty acids esterified with cholesterol are also utilized has not been entirely excluded. Hence it is of interest to record that Voris, Ellis & Maynard (1940) have described a method for the estimation of triglycerides, by determination of glycerol, by means of which in three out of

four A.-V. experiments on a lactating cow they were able to demonstrate a considerable uptake of neutral fat by the udder, perhaps more convincingly than had before been possible. One may remark that this method does not appear to have been put to subsequent use for mammary gland studies; it may be that the preference shown (Shaw & Petersen, 1940; Shaw *et al.* 1942) for the less specific Allen (1938) method may be due to its convenience.

Balance experiments (Shaw & Petersen, 1938*a*, 1940; Shaw *et al.* 1942) have given their proponents no grounds for believing that the rate of absorption of blood fat is insufficient to account for the fat secreted in the milk.

Absorption of blood fat by the lactating udder appears to be characterized by one feature which is of interest and perhaps a little surprising. Shaw & Petersen (1940), studying A.-V. blood fat differences in the cow in relation to the time since milking, found that the uptake, negligible immediately after milking, gradually rose to a maximum over a period of some hours. They concluded that the mammary gland cells only become fully permeable to neutral fat when the glandular tissues are distended with secretion. The implication of these findings in relation to the validity of mammary gland fat balance computations has been discussed on p. 318 *et seq.*

Certainly the possibility of the mammary alveolar cells being permeable to neutral fat is not inconsistent with modern views on the mechanism of fat absorption from the intestine (see Frazer, 1946) though it is not clear why the fat permeability should increase with the degree of secretory distension of the tissues. The actual process of absorption may involve lipolysis, since not only is lipase present in the mammary tissue (Kelly, 1942) but, according to Kelly & Petersen (1939), free fatty acids can be demonstrated histochemically in the basal portion of the epithelial mammary cell.

The only alternative modern theory of the origin of the milk fat which has been at all extensively canvassed is the view that some proportion of it may be formed in the mammary gland itself from carbohydrate. This theory, first proposed by Graham Jnr., Houchin, Peterson & Turner (1938) because they obtained a high R.Q. for the udder of the lactating goat, is discussed more fully in the following section together with yet other possibilities in connexion with the origin of the lower fatty acids of milk fat.

(2) *Origin of the short-chain fatty acids of milk fat*

The origin of the short-chain acids (C_4–C_{14}) which are a characteristic feature of the milk of herbivores and particularly ruminants (see Hilditch, 1947) has been the subject of much speculation. This problem is not only of interest intrinsically but also because the origin of the short-chain acids may be only a facet of the larger question of the mode of formation of milk fat and the solution of this particular problem may mean at least a partial solution of the more general one also.

There seem to be three main possibilities: (*a*) they may be formed by degradation of long-chain fatty acids in the mammary gland by a process of oxido-reduction; (*b*) they may be synthesized from carbohydrate; or (*c*) they may arise by a process of synthesis from short-chain acids transported to the mammary gland by the blood.

(*a*) The view that the short-chain acids are formed by degradation of long-chain acids in the mammary gland was first advanced by Hilditch and his collaborators (for summary see Hilditch, 1937, 1947) on the basis of extensive chemical studies on the glyceride structure of milk fat, particularly from the cow, in which regular relationships emerged between the proportion of fully saturated glycerides to total glycerides on the one hand and the proportion of saturated acids to total fatty acids on the other. It was suggested that the lower fatty acids of bovine milk fat arise by oxidative degradation of pre-formed oleo-glycerides beginning at the end of the carbon chain remote from the carboxyl group (a type of ω-oxidation) so that one molecule of oleic acid would give rise to one molecule of short-chain acid. In order to give rise to saturated acids it would of course be necessary that oxidation should be followed by reduction. In support of this theory it was later shown (Hilditch & Paul, 1936; Hilditch, Paul, Gunde & Maddison, 1940) that milk fat contains lower unsaturated acids, decenoic, dodecenoic, tetradecenoic and hexadecenoic, in which the position of the double bond relative to the carboxyl group is the same as in oleic acid, suggesting that these acids represent degradation products of oleic acid which have escaped complete saturation. Further supporting evidence came from studies of the effect of cod-liver oil feeding on the composition of the milk fat of the cow (Hilditch & Thompson, 1936). This treatment decreases the milk fat percentage but the relative proportions of oleic acid and short-chain acid molecules are changed in such a way that the total daily yield of the latter is decreased much more than that of the former which in fact may be hardly changed (Hilditch, 1937). The interpretation here is that highly unsaturated acids present in the cod-liver oil pass into the gland and poison the enzyme system responsible for the oxidative degradation of the oleic acid chain, so that the molar proportion of oleic acid is increased at the expense of that of the short-chain acids. The results of Smith & Dastur (1938), who found a similar increase in the proportion of oleic acid in bovine milk fat during inanition coupled with a decrease in the short-chain acids are also, as these authors point out, consistent with such a view.

It has been claimed that A.-V. studies also have provided evidence that fat is oxidized in the mammary gland. Shaw & Petersen (1938*a*) reported that the uptake of blood fat (Allen's method) was more than sufficient to provide the milk fat and concluded that the excess could be accounted for if it be assumed that a proportion is oxidized to the stage of short-chain acids as postulated by Hilditch. If as Shaw & Petersen (1940) state, lymph arising from the mammary gland contains appreciable amounts of calcium but very little fat, then the actual uptake might be considerably greater than the observed value (see p. 318), and though in this later paper they infer from the observed A.-V. differences in fat and calcium merely that 'the quantity of blood fat used by the gland is sufficient to account for all of the milk fat' it may well be that in actual fact the uptake was considerably underestimated. Shaw *et al.* (1942) concluded from A.-V. experiments on the cow, in which the blood/milk ratio was calculated from calcium uptake, that the uptake of blood fat was sufficient to account for only about 93% of the milk fat secretion, which would

appear to leave no margin for oxidation. Here again, however, the estimate must be low because no account was taken of the lymph.

(*b*) The theory that the short-chain acids, in particular, of milk fat are formed in the gland from carbohydrate was first explicitly stated by Reineke, Stonecipher & Turner (1941). The evidence in favour of this theory consists in the high R.Q. of the lactating gland, discussed in an earlier section (p. 323 *et seq.*), coupled with a decrease of the R.Q. below unity during inanition which also depresses the short-chain fatty acids of the milk. The R.Q. is of course not decisive as evidence of the nature of the metabolic processes proceeding in an organ, as proponents of the fat oxido-reduction theory are not slow to point out, usually quoting Soskin (1941) in support. It seems, however, difficult to escape the conclusion that mammary gland slices of some species are *in vitro* able to synthesize fat from glucose (see p. 324) and if *in vivo* the gland is able to carry out a similar process then it would seem reasonable to assume that if a proportion of the higher fatty acids of milk fat is synthesized in this way, the lower acids might represent by-products or intermediates of such a synthesis. Smith & Dastur (1938) indeed suggest that the changes in the relative proportions of oleic and short-chain acids in the milk fat secreted by starved cows are as consistent with the theory that oleic acid is synthesized from carbohydrate as with the theory of Hilditch. They point out that the lowered rate of milk fat production characteristic of inanition might well establish conditions that are favourable for such a synthesis being more complete than it would be when fat was being formed at its normal rate. However, the low R.Q. found by Folley & French (1948*c*, 1949*a*, *b*) for slices of cow and goat udder in presence of glucose (see p. 324) tends to throw doubt on the importance of fat synthesis from carbohydrate in the ruminant udder, particularly since the high R.Q. *in vivo* may be susceptible of other explanations (see p. 342 *et seq.*).

(*c*) There remains for discussion the possibility that the lower fatty acids of milk fat arise from short-chain acids (C_2–C_4) circulating in the blood. Acetoacetic acid and β-hydroxybutyric acid, well-known intermediates in fat metabolism, may be considered first in this connexion. Arterio-venous studies (Shaw & Knodt, 1941*a*; Shaw, 1942) indicate no utilization of acetoacetate by the lactating bovine udder even in cases of ketonaemia (Shaw, 1942) and studies on the perfused isolated bovine udder (Shaw & Petersen, 1943) lead to the same conclusion. On the other hand, an appreciable uptake of β-hydroxybutyric acid by the lactating bovine udder has been reported (Shaw & Knodt, 1941*a*; Shaw, 1942; Shaw, Powell Jnr. & Knodt, 1942) and confirmed in work on the perfused preparation (Shaw & Petersen, 1943). The A.-V. difference is increased in ketonaemia (Shaw, 1942), but the presumed increased utilization does not appear to be connected with the low blood level of glucose characteristic of ketosis because the gland perfused with blood containing large amounts of β-hydroxybutyrate showed an abnormally high utilization even when the perfusion blood was hyperglycaemic. Shaw & Knodt (1941*a*, *b*) suggested that the short-chain acids of bovine milk fat might arise by synthesis from β-hydroxybutyrate, a process which would increase the R.Q. and in this respect fit in with the

experimental findings. They calculated from balance experiments that the uptake observed by them was sufficient to account for the short-chain acids up to C_{14}. Supporting evidence was considered to be provided by the finding, in the cow, that fasting or feeding cod-liver oil decreased both the mammary gland R.Q. and the proportion of short-chain acids in the milk fat (Shaw & Knodt, 1941*b*). However, the proportion of short-chain acids in the milk of the cow decreases in ketosis at any rate when there is inanition (Shaw, 1941*b*; Shaw *et al.* 1942), while the uptake of β-hydroxybutyrate appears to increase, which would seem to exclude the possibility that the short-chain acids of milk fat arise from this substance.

A further, most interesting possibility involving synthesis from a two-carbon compound remains to be considered. It is noteworthy that short-chain fatty acids are only found in appreciable amounts, as far as we know, in the milk fat of herbivores and particularly ruminants (see Hilditch, 1947). This may assume added significance when considered in the light of recently acquired knowledge of rumen digestion (see Elsden & Phillipson, 1948 for review). The rumen may be regarded as a fermentation chamber in which micro-organisms break down cellulose and other plant polysaccharides, the principal end-products being lower fatty acids, chiefly acetic and some propionic and butyric, which are absorbed as such and utilized by the body. Recent studies by Reid (1948) and McClymont (1948), who have found acetate levels of the order of 10 mg. % in the arterial blood of sheep and cows respectively, indicate that products of rumen digestion, such as acetate and propionate, probably reach the udder in amounts sufficient to play a significant role as milk precursors. Such possibilities will no doubt increasingly occupy the attention of investigators in the mammary field; already there have been a few preliminary studies. The possibility that acetate, arising from the rumen, may serve as a substrate for the synthesis of fat by the mammary gland, in the course of which the short-chain acids might appear as by-products or intermediates (put forward by Folley, 1945 and also discussed by Owen, 1946), appears particularly attractive in view of the demonstration by Rittenberg & Bloch (1945), using labelled acetate, that fatty-acid chains can be built up from acetate in the mammalian body.

Fasting very quickly lowers the proportion of short-chain acids in the milk fat (e.g. Smith & Dastur, 1938) and the fasted ruminant would seem a suitable subject for attempts to discover the precursors of the short-chain fatty acids by direct means. In 1946, Folley & Malpress (unpublished, but see Malpress, 1946) attempted to test acetate as a precursor of short-chain fatty acids by slow intravenous (jugular vein) infusion of amounts of the order of 50 g. neutralized sodium acetate, but were unsuccessful in raising by this means the depressed Reichert-Meissl and Polenske values to normal levels. Somewhat similar experiments on cows, which must have been carried out at about the same time, also with negative results, were described by Mann & Shaw (1947). In addition to acetate (1984 g. over 22 hr.) other possible precursors, glucose, oleic acid, butyrate, were tested in infusions into the mammary vein which sometimes lasted up to 31 hr. A positive result was in one case obtained with butyrate but could not be repeated.

More recently (Folley & French, 1948*c*, 1949*a*, and further unpublished work), results have been obtained in this laboratory which may provide the first evidence for the synthesis of ruminant milk fat from acetate. These experiments, carried out on lactating mammary gland slices and already referred to on p. 324, indicate that goat (and cow) gland will utilize acetate as sole substrate with a high R.Q. (1·2), but will not give a high R.Q. with glucose, a substrate readily utilized by tissue from all non-ruminants studied; rat and mouse tissue, on the other hand, will hardly metabolize acetate under these conditions. It would appear probable that in these experiments the goat udder slices were synthesizing fat from acetate at an appreciable rate since such a synthesis would give a high R.Q. Thus on the assumption that for every molecule of acetate oxidized another is used for fatty acid (say butyrate) synthesis it is possible to write the following equation corresponding to an R.Q. of about 1·3:

$$4\,CH_3COOH + 3O_2 = CH_3.CH_2.CH_2.COOH + 4CO_2 + 4H_2O.$$

One can of course write an equation giving any R.Q. up to infinity but this may serve to illustrate the type of overall reaction which could help to give the R.Q. found experimentally.

These results, if confirmed by further work, may mean that the ruminant mammary gland is better adapted for the synthesis of fat from acetate, a known product of rumen fermentation, than from glucose which, however, judging by the *in vitro* R.Q. seems to be well utilized for milk fat synthesis by non-ruminants. They are in harmony with the theory that, in ruminants at least, part of the milk fat is synthesized from acetate—a process which would explain the high *in vivo* R.Q.—and if this is so it would seem probable that the lower fatty acids in particular arise in this way.

Evidence in harmony with the probable formation of the short-chain acids of ruminant milk fat from acetate has also been obtained by McClymont (1948). In A.-V. studies on the cow he has observed A.-V. acetate differences, for the lactating udder, amounting to 50–80% of the arterial level, and to about 30% for the dry udder, i.e. differences of 2–8 mg. % depending on the time since feeding. The depression in the milk short-chain acids during fasting was associated with lowered arterial acetate levels and decreased A.-V. differences. Intraruminal infusion of acetic acid or intravenous infusion of sodium acetate failed to increase the lowered Reichert-Meissl values despite the maintenance of normal or supernormal levels of acetate in the arterial blood and of acetate uptake (i.e. A.-V. differences) by the mammary gland. In explanation of such negative results McClymont suggests that fasting may cause a change in endocrine balance resulting in a shift of emphasis from fat anabolism to catabolism. In this connexion it may be recalled (see p. 325) that in the rat, partial inanition seems to abolish net fat synthesis by mammary gland slices *in vitro* even in presence of adequate concentrations of glucose (Folley & French, 1949*b*).

In sum it can be said that existing evidence indicates that milk fat is formed by more than one process proceeding concurrently. Part of it undoubtedly arises from the blood fat as the appearance in the milk of iodine-labelled dietary fat (Aylward,

Blackwood & Smith, 1937) coupled with the generally agreed A.-V. difference in blood fat would appear conclusively to prove; in the ruminant a proportion is probably synthesized from metabolites arising from the rumen, while in no species is the possibility that some milk fat is formed from carbohydrate (or indirectly from protein) by any means excluded.

VII. NITROGEN METABOLISM

(1) *Precursors of milk protein*

Arterio-venous balance experiments (reviewed by Folley, 1940) have indicated the inadequacy of the small but generally observed A.-V. differences in free amino-acids (more recently confirmed by unpublished observations of Bottomley & Folley and by Shaw, 1946) to account quantitatively for the synthesis of the milk proteins, casein and lactalbumin. The discrepancy is so marked—something like twice the observed uptake would be required—that the possibility of shortcomings, on the quantitative side, of the A.-V. technique obscuring the issue can almost be discounted. Therefore in the absence of any other non-protein nitrogenous fraction known to be absorbed by the gland, and none has so far been identified, one is forced to look to some fraction of the blood plasma proteins as the main source of materials for the synthesis of milk protein.

Earlier work (Graham Jnr., Peterson, Houchin & Turner, 1938; Reineke, Peterson, Houchin & Turner, 1939) indicated rather complicated and obscure changes among blood protein fractions during passage of the blood through the mammary gland, which were interpreted as evidence of the transformation of blood protein to milk protein. Since then the work of Reineke, Williamson & Turner (1941*b*), who observed an uptake of protein-bound carbohydrate by the udder of the lactating goat, has provided further evidence in favour of earlier suggestions from the same laboratory that the active gland absorbs proteins, probably belonging to the globulin fraction, from the blood. The fact that Reineke, Williamson & Turner (1941*b*) and Shaw *et al.* (1942) found no uptake of amino-acids by the udder of the lactating goat and cow respectively during inanition, even though some milk was still being secreted, is a further indication that blood amino-acids are not essential for the synthesis of milk protein.

Apart from these few investigations, no further progress with the difficult problem of the mode of synthesis of milk protein has been made since the subject was last reviewed in this journal. If the implication of some fraction of the plasma proteins as precursor is taken as the most probable conclusion to be drawn from present knowledge, the problem for future attack by modern methods of protein chemistry would appear to resolve itself into two possibilities, first that the blood protein may be broken down in the gland into small fragments (peptides and amino-acids) from which the molecules of the milk proteins are assembled; secondly, as the investigators cited above have suggested, that the latter arise by reshuffling of relatively high-molecular proteinaceous fragments.

(2) *Deamination in the mammary gland*

The liver is still generally believed to be the only organ capable of forming urea from amino-acids (e.g. see Peters & Van Slyke, 1946). There is now, however, a fair amount of evidence which suggests that this process also proceeds to some extent in the lactating mammary gland at any rate in some species. This may be summarized as follows:

(*a*) Negative A.-V. urea differences, indicating the formation of urea by the goat udder, have been reported by Graham Jnr., Houchin & Turner (1937); Graham Jnr., Houchin, Peterson & Turner (1938) and Reineke, Peterson, Houchin & Turner (1939).

(*b*) A small but definite uptake of blood amino-acids by the lactating ruminant udder has been observed quite uniformly (for references see Folley, 1940, in addition to preceding section). Since, as we have seen, there is little reason to believe that the absorbed amino-acids are incorporated into milk proteins it seems likely that, as Graham Jnr. (1937) first suggested, they are deaminated and the residues used in the fat or carbohydrate metabolism of the gland.

(*c*) The mammary gland contains arginase (Shaw & Petersen, 1938*b*; Folley & Greenbaum, 1946, 1947*a*) an enzyme quite generally believed to be a component of the urea-forming mechanism of the liver.

On the basis of the conception of the lactating mammary gland as an organ which, like the liver, is capable of deaminating amino-acids with the formation of urea, Folley & Greenbaum (1947*a*) have interpreted the striking increase in the concentration of arginase in the mammary gland, beginning at about the fifth day of lactation, which they observed in the rat (Fig. 1), as indicating that in this species gluconeogenesis from protein proceeds to an increasing extent in the mammary gland as the demands of lactation increase. This interpretation involves the assumption, presumably equally applicable to the liver and mammary gland, that arginase present in a urea-forming tissue is synthesized, by a mechanism at present unknown, in response to some stimulus such as an increase in the rate of presentation of amino nitrogen for incorporation into urea. It has been suggested (Edlbacher & Merz, 1927; see also Baldwin, 1936) that in some organs arginase may be related to cellular multiplication, but this alternative possibility could hardly be applicable to the mammary gland arginase, since mitosis in the lactating gland is relatively rare (e.g. see Weatherford, 1929).

The effects of adrenalectomy on tissue arginase levels are of interest in the present connexion. Adrenalectomy depresses the mammary gland arginase level in the rat (Folley & Greenbaum, 1946), an effect which is not merely a secondary effect of post-operative anorexia because the same result is obtained in pair-feeding experiments (Folley & Greenbaum, 1947*b*, 1948*b*). If it is accepted that gluconeogenesis from protein can proceed in the mammary gland and that changes in the mammary gland arginase content indicate changes in the rate of this process, this finding may throw some light on the partial inhibition of lactation which follows adrenalectomy (see

Cowie & Folley, 1948). It is now generally accepted (see Ingle, 1944) that adrenal cortex hormones, probably the 11-oxygenated steroids, exert a direct effect on the rate of gluconeogenesis from protein. Hence in species, such as the rat, in which gluconeogenesis appears to be an important element in the metabolism of the fully lactating mammary gland, the decline in the rate of this process which follows adrenalectomy would no doubt adversely affect lactation. It may well be that the relatively small, but nevertheless distinct, decrease in milk yield, over and above the effect ascribable to post-operative anorexia, which Cowie & Folley (1948) have shown to result from adrenalectomy in the rat, is due to suppression of nitrogen catabolism in the mammary gland itself.

In addition, and probably in all species, the adrenals may affect lactation via the liver. In the rat, lactation from the outset seems to demand an increase in the liver arginase level above that of pregnancy (Folley & Greenbaum, 1947*a*), possibly indicative of the formation of extra carbohydrate from protein to satisfy the demands of the mammae for milk precursors. Since adrenalectomy, as is well known, would decrease the capacity of the liver for gluconeogenesis—and the depression in liver arginase following adrenalectomy (Fraenkel-Conrat, Simpson & Evans, 1943; Folley & Greenbaum, 1946, 1947*b*, 1948*b*) is in accord with this— it is obvious that the adrenalectomized animal is in an unfavourable position for the supply of carbohydrate to the mammary gland. Incidentally, it seems unlikely that the effect of adrenal cortex hormones on gluconeogenesis is mediated through a direct action of these hormones on arginase; available evidence indicates that the changes in liver and mammary gland arginase which follow adrenalectomy result from alterations in the rate of deamination of amino-acids (Folley & Greenbaum, 1949).

However, deamination processes occurring in the mammary gland apparently do not play as important a role in lactation in all species as in the rat if the concentration of arginase in the mammary tissue during lactation may be taken as any indication. While the mouse appears to be similar to the rat as judged by the mammary gland arginase level (Table 3), the enzyme concentrations in the mammary tissues of herbivores are much lower and the enzyme is for all practical purposes absent from the gland of the rabbit in early lactation. Table 3 shows that the goat and cow must be numbered among forms in which the mammary arginase levels are relatively low, a fact which is difficult to reconcile with the finding that the lactating goat udder produces urea. If further studies serve to multiply such apparent anomalies the role of arginase in the mammary gland as discussed above would have to be reconsidered.

VIII. SOME GENERAL CONCLUSIONS

It may be useful in conclusion to point out one or two general concepts which emerge from the foregoing appreciation of the present position in the field covered by this article. First, it is becoming increasingly evident that we must reckon with the possibility that major milk constituents may be formed by more than one process simultaneously. This is almost certainly so in the case of milk fat which at once may

originate from blood fat on the one hand, and on the other by synthesis from carbohydrate, or, in the case of ruminants, from metabolites formed in the rumen. Secondly, we cannot afford to overlook the accumulating evidence of differences between various animal forms as regards the contribution of various types of metabolic process to the total metabolism of the mammary gland. Thus the deamination processes which seem so important in the rat and mouse appear to be much less prominent in the guinea-pig, rabbit, goat and cow, among which, moreover, the ruminants demand special consideration by virtue of various interesting possibilities in connexion with the utilization by the udder of acetate and perhaps other products of rumen fermentation.

IX. SUMMARY

1. The arterio-venous technique, hitherto favoured for mammary gland studies, is subject to drawbacks which make it unsuitable for quantitative work, as many conflicting results attest. This technique in its present form is capable of yielding only the broadest qualitative conclusions. It is claimed that one source of uncertainty is minimized by the use of anaesthetized animals. Studies on the isolated perfused udder and surviving tissue slices have given useful results; tracer isotopes, so far hardly used in this field, may solve many outstanding problems.

2. Mammary slices respire fairly actively compared with other tissues. In the rat there is a marked increase in respiration at parturition, correlated with hormonally controlled changes in mammary gland metabolism, and the respiration further increases with increasing milk yield. Glucose and mannose are the only sugars so far found which increase the oxygen uptake of rat mammary gland slices above endogenous values.

3. Studies *in vivo* and *in vitro* alike indicate a high respiratory quotient for the lactating gland which may mean the synthesis of fat from oxygen-rich precursors. Studies *in vitro* suggest that in the non-ruminant the fat precursor may be carbohydrate while the ruminant gland probably synthesizes fat from acetate. Non-lactating gland gives an R.Q. less than unity and the hormonally controlled events associated with parturition are accompanied by a rapid increase in the R.Q. to values greater than unity.

4. The source of energy of the active gland is not yet clear; there is evidence that carbohydrate, long-chain fatty acids, β-hydroxybutyric acid and acetate can be oxidized.

5. Mammary gland slices show appreciable anaerobic glycolysis (acid production) which is decreased in presence of oxygen. It is possible that part of the aerobic acid production may be due to formation of citric acid or fatty acids.

6. Enzymes recently detected in mammary tissue comprise desoxyribonuclease, catalase, xanthine dehydrogenase, and acid phosphatase. Most attention has been given to the alkaline phosphatase and arginase, both of which occur abundantly in lactating gland in certain species.

The concentration of alkaline phosphatase in the rat mammary gland increases rapidly during late pregnancy to a constant high level which is maintained throughout

lactation, indicating that the enzyme is concerned with mammary function rather than growth. Histoenzymatic studies have suggested that this enzyme is mainly localized in the myoepithelium and capillary endothelium, there playing a role associated with transport of metabolites, rather than in the secretory epithelium, but this needs further confirmation.

Changes in the arginase levels of the mamma in the rat during lactation suggest that deamination processes probably associated with gluconeogenesis from protein or amino-acids play an important role in mammary metabolism in this species. The arginase content of the mamma is low in herbivores (including ruminants) so that deamination in the mammary gland must be less prominent in these forms.

7. Present evidence indicates that glucose is the principal blood precursor of lactose, though other hexoses, absorbed from the blood as glycoprotein, may supplement the carbohydrate supplies of the udder. There is no evidence for the absorption of blood lactate and pyruvate by the mammary gland.

The role of glycogen, present in disputed amounts in mammary tissue, in lactose formation, is not clear; it may be an obligatory intermediate or merely a reserve of carbohydrate. Mammary glycogen levels can be experimentally increased by perfusion of the isolated gland with hyperglycaemic blood, particularly in presence of insulin, or by weaning.

The mode of formation of the galactose moiety of the lactose molecule is still obscure. There is no evidence that galactose transported to the mamma in the blood is necessary for lactose synthesis, and its formation in the free state from glucose in the gland, prior to condensation with glucose to give lactose, is contra-indicated by the available evidence. The possibility that glucose is transformed enzymatically to a galactose phosphate before condensation is considered and a tentative scheme of biological lactose synthesis on this basis is proposed.

8. Part of the milk fat undoubtedly arises from neutral blood fat, probably from the glyceride fraction, but the high R.Q. of the lactating gland indicates that some at least of the fat may be synthesized from oxygen-rich compounds. The facts that cow and goat udder slices apparently do not utilize glucose with high R.Q. as tissues from other species do, but unlike other species utilize acetate with high R.Q., suggest the possibility that the ruminant udder may synthesize fat from acetate. If so it seems likely that the short-chain acids of ruminant milk may arise in this way, rather than from carbohydrate or by partial oxidation of long-chain fatty acids as previously suggested.

9. Very little is known about the mode of synthesis of milk protein. The amount of free amino-acids absorbed from the blood by the udder is too small to account for the milk protein and it is probable that some fraction of the blood proteins is transformed into the proteins of milk.

10. It appears that the mammary gland, like the liver, can deaminate amino-acids with the production of urea—at least in some animals, and it is likely that in forms in which the mammary gland arginase levels are elevated during lactation, the process is an important element in the metabolism of the lactating gland. In such forms the

partial inhibition of lactation following adrenalectomy may be due to depression of gluconeogenesis from protein in the mammary gland. A curious anomaly is the low concentration of arginase in the udder of the goat, the only animal in which direct evidence of urea production by the mammary gland has yet been obtained.

11. In the rat and probably other animals lactation demands an increase in the liver arginase level above that of pregnancy, probably indicative of increased gluconeogenesis from protein to meet the requirements of the mammary gland for carbohydrate.

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ADDENDUM

Work carried out since this review was written has provided further information about the mechanism of milk fat formation. Folley & French (unpublished) have shown that lactating mammary gland slices from yet another ruminant, the sheep, will, like those from cow and goat, utilize acetate *in vitro* with high R.Q. The theory of the utilization of acetate for milk fat synthesis by the mammary gland, thus strengthened, has received independent and powerful (perhaps decisive) support from tracer experiments of Popják & Beeckmans (1949*a*), who found that glyceride fatty acids, isolated from the mammae of pregnant rabbits fed acetate labelled with ^{14}C, contained outstandingly high concentrations of isotope. An important point was that the mammary fat was more radioactive than that of the liver and so could not have been derived from it. A high rate of incorporation of deuterium into the neutral fat fatty acids of the mammary gland could also be observed (Popják & Beeckmans, 1949*b*). Fractionation of the total glyceride fatty acids from the mammae of these animals (Folley, French & Popják, unpublished) revealed the presence of considerable amounts of short-chain (volatile) acids, both water soluble and insoluble, and the ^{14}C content of both volatile fractions was much higher than that of the non-volatile fraction. These results point unmistakably to the utilization by the rabbit mammary gland of acetate (or some two-carbon unit derived therefrom) for the synthesis of milk fat, a process which appears to begin in late pregnancy. Moreover, they seem to settle conclusively the disputed problem of the origin of the short-chain acids of milk fat; there is little doubt that these acids are formed by condensation of two-carbon chains. As present in mammary tissue they probably represent intermediate stages in the synthesis of long-chain fatty acids, but as they occur in milk, i.e. withdrawn from the site of fat synthesis, they are probably better regarded as by-products of fat synthesis.

Though this tracer work shows that rabbit mammary tissue can utilize acetate for milk fat synthesis *in vivo*, it has been found (Folley & French, unpublished) that rabbit mammary slices (like those from other non-ruminants) utilize very little acetate *in vitro* and the R.Q. is low. That is, using the R.Q. as criterion, it has not been possible to demonstrate net fat synthesis from acetate alone by rabbit mammary tissue *in vitro*. However, recent experiments by Folley & French (1949*a*) do much to

harmonize the tracer and tissue slice results in the rabbit. Following the work of Bloch & Kramer (1948), who reported that glucose or pyruvate increases the incorporation of labelled acetate into fatty acids by liver slices, it has been found that in presence of small concentrations of glucose, rat and rabbit lactating mammary slices markedly utilize acetate with R.Q. always greater than unity and usually of the same order as with glucose alone. The maintenance of the R.Q. above unity and the fact that though Q_{O_2} was increased above the values for glucose alone the increment was insufficient to explain the increased acetate utilization in terms of oxidation, leaves little room for doubt that acetate is incorporated into fat under these conditions. Mammary slices from non-ruminants can thus utilize acetate *in vitro* for net fat synthesis provided glucose is present. The latter perhaps provides glycerol for the formation of glycerides, a process which might well favour fatty acid synthesis. This effect of glucose on acetate utilization was even more marked with lactating sheep tissue, a fact which emphasizes the metabolic difference between ruminant and non-ruminant mammary tissues, since it means that one must resist the temptation to explain the differences in the ability of mammary tissue from various species to utilize acetate with high R.Q. in terms of differences in carbohydrate stores (see p. 334).

Bloch & Kramer found that insulin increased the effect of pyruvate but not of glucose. On the other hand, Folley & French found that their glucose effect was increased by insulin provided *o*-cresol, added as a preservative to commercial insulin solutions, was present. The explanation of the apparent difference was that *o*-cresol increases the glucolysis of the slice, thus providing lactate and hence pyruvate, on which the insulin can act.

With regard to the biological synthesis of lactose, Folley & French (1949*b*) have drawn attention to the possible significance of recent work (Friedmann, 1949), which shows that, aside from glucosamine, the carbohydrate moiety of serum glycoprotein in the horse consists of an equimolecular mannose-galactose complex. Since mannose and glucose are inter-convertible by the mammary gland (at least in the rat) as shown by the fact that these are the only sugars, so far found, which increase the respiration and R.Q. of mammary gland slices (pp. 322 and 324), the possibility that lactose may, partly at least, arise by transformation of a mannose-galactoside derived from blood glycoprotein absorbed by the mammary gland (p. 332) deserves consideration, in spite of the fact that, as pointed out on p. 335, there is as yet no evidence that galactose brought to the gland in the blood is necessary for lactose synthesis. Here we are irresistibly reminded of the prescience of Hammond (1913), who long ago suggested that lactose and casein may arise from a glycoprotein precursor.

In conclusion, one or two miscellaneous observations should be quoted. Massart, Peeters & Quenon (1948) report that an acridine derivative which, when infused into the sheep's udder, inhibits lactation, has no effect on the respiration of udder tissue. In the tracer field, Kleiber, Smith & Ralston (1948) report a study of the excretion of ^{32}P in the milk of cows given labelled phosphate. Casein labelled with radioactive P was isolated from the milk, confirming the findings of Aten Jnr. & Hevesy

(1938). This study appears to be an 'armed reconnaissance' in preparation for a further and perhaps more rewarding attack on mammary gland problems by the tracer technique.

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CHEMICAL MUTAGENESIS

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I. INTRODUCTION

Attempts to induce genic changes by chemical means go back to the early days of modern genetical research. Even when Muller's discovery (1927) of the mutagenic effects of X-rays had opened up a new and exceedingly fruitful field for mutation work, which attracted many geneticists, the search for chemical mutagens was not abandoned. Although it was unlikely that a chemical substance would exceed or even equal high-energy radiation in quantitative efficiency, chemical mutagens might be expected to offer other advantages. It seems fair to presume that the gene is a chemically definable molecule, and that mutation is a chemical process. Therefore known chemical substances appear a more appropriate tool for studying the nature of the gene and of mutation than drastic and indiscriminating physical agencies. Moreover, it seemed not impossible that chemical substances might be found which are able to single out individual genes for mutagenic effects; the outstanding theoretical and practical importance of such a possibility is obvious.

Earlier attempts to induce mutations by chemical means have been reviewed briefly by Timoféeff (1934) in *Biological Reviews*. A few other references can be found in Auerbach (1941). In addition, there is a paper by Law (1938) on experiments with colchicine and a number of salts; also claims by Gershenson (1939), that the mutation rate of *Drosophila* is raised by the addition of thymonucleic acid to the food of the larvae, which, however, could not be confirmed by either Rapoport (1940) or Muller (1941). Russian experiments between 1934 and 1940 have been briefly reviewed by Dobzhansky (1941, p. 46).

These earlier experiments will not be discussed here except when they have a bearing on the problems and possibilities opened up by the more recent discoveries.

None of the pre-war experiments brought satisfactory proof of the existence of chemical mutagenesis. It is true that in some of them the difference between mutation rates in treated and control series was statistically significant; but in each case the mutation rates were so small that confirmatory data, obtained under the most rigidly controlled conditions (see § II), would be required to establish the reality of the effect.

In recent years a great number of substances have been found capable of arresting cell division ('mitotic poisons'), or of causing chromosome abnormalities such as stickiness, fragmentation and translocation. These results will only be considered when the observed effects have been shown or may be expected to lead to genetic consequences.

II. GENERAL METHODOLOGICAL REMARKS

Timoféeff in 1934 gave a list of requirements for mutation work. They are: (1) genetic purity of the material, (2) sufficiently large numbers in both controls and treated material, (3) suitable genetical methods for the detection of newly arisen mutations, (4) analysis of the detected variants as to their nature (whether non-heritable, cytoplasmic, chromosomal, genic), and (5) some knowledge of the manner in which the agent used can act on the germ cells of the treated organism (penetration; direct or indirect action). Of these requirements, which have to be fulfilled in every type of mutation work, the fifth acquires particular importance when chemical substances are used. Whereas difficulties of penetration play a role only with a few types of radiation (ultra-violet, α-rays), they are all-important in work with chemicals. This was demonstrated clearly in an analysis of the sensitivity of *Drosophila* males to the action of mustard gas (Auerbach & Robson, 1942*b*, 1947*a*). When mustard gas-treated males are mated to untreated females, hatchability of the eggs is reduced to an extent which depends not only on the strength of the treatment, but also on the type of males used. When males of two different strains are exposed together to the same dose of mustard gas, hatchability in the progeny of one strain may be reduced to zero, while in the progeny of the other it is hardly affected. When, however, spermatozoa of the most and the least sensitive strains were exposed, not in the males themselves, but after copulation in the seminal receptacles of females of a third strain, the difference in sensitivity had completely disappeared; thus it must have been caused entirely by strain differences in penetrability of mustard gas to the germ cells of the males. Whether or not a similar cause is responsible for the gene-controlled sensitivity of *Drosophila* to carcinogenic aerosols which Demerec has reported (1947*a*, *b*) has not yet been determined. Thus, failure of a substance to produce genetical effects may simply be due to its inability to reach the chromosomes, or to the fact that in doing so it interacts with the surrounding cytoplasm in a way which prevents survival of the affected cell. Moreover, the organism as a whole may be killed by concentrations of a toxic mutagen which are insufficient for effective penetration to the chromosomes of the germ cells. On the other hand, a positive result need not be due to a direct effect of the tested substance on the chromosomes. Treatment may

have increased mutation rate in a more or less indirect way: by prolongation of development, by disturbance of general or cellular metabolism, by changes in the permeability of membranes, by the production of a cytoplasmic constituent which in its turn is mutagenic, etc. In such a case, the same result might be obtained by unrelated substances or even by quite different means; also, the effect would be expected to be highly dependant on environmental and physiological conditions. This is especially true when the method chosen is to mix the substance with the food. Not only is it very probable that substances mixed with the food will reach the germ cells only after transformation in the course of digestion and metabolism; it is also likely that the chemical may undergo transformation even before ingestion, by interaction with normal food constituents, and with micro-organisms present in the food. An example of such a kind of interaction was found in unpublished experiments with acetylamino-fluorene. *Drosophila* which has been reared on food mixtures containing this substance shows pronounced phenotypical effects when seeding of the culture vials with live yeast has been omitted; in the presence of a living yeast population—or of a rich bacterial flora—the effects are very slight or absent. Wyss, Stone & Bennett Clark (1947) and Wyss, Bennett Clark, Haas & Stone (1948) were able to produce bacterial mutations by treatment of the culture medium with hydrogen peroxide, but not by direct treatment of the cells with this substance.

The work with mustards (see below) has shown that at least this group of chemicals may produce delayed mutations in chromosomes which have been derived from treated chromosomes. Detection of these late mutations requires an extension of the usual methods. Without this precaution, the mutagenic potency of certain substances can be underestimated and may even remain undetected.

Radiation work has accustomed geneticists to expect a positive correlation between dose and effect. This is not necessarily true for chemical mutagens; in fact, the work of Levan & Hin Tjio (1948*b*) has shown that chemically induced nuclear effects in plants depend qualitatively on concentration, certain types of effect being produced within certain limits of concentration. This experience should caution us against the usual method of testing chemical substances in the highest concentration tolerated by the organism, disregarding the possibility that a substance which fails to induce mutations in this limiting concentration may yet do so at a lower one.

A peculiar difficulty arises when a quantitative comparison between the results of different experiments is desired. The necessity to restrict such comparisons to material of exactly the same type is discussed in § III; but this removes only part of the difficulty. Differences in penetration and probably also in secondary reactions inside the organism, and, in feeding tests, inside the medium, make an estimate of the amount which reached the tested nuclei highly uncertain. Doses which are equal in terms of chemical and physical constants may yet result in different concentrations in the tested cells. Various incidental circumstances, too, may play a role. Thus, activity during exposure to mustard gas increases the sensitivity of *Drosophila* males (Auerbach & Robson, 1947*a*); for the percentage of hatchability in the progeny of treated males was improved by removing the wings of the males before exposure

to mustard gas. Until accurate methods for determining the concentration inside the tested cells are available, dosage estimates will have to be based on some nuclear effect. Auerbach & Robson (1947*a*), in work with mustards on *Drosophila*, assessed the effective dose in terms of frequency of induced sex-linked lethals. Two doses were called 'mutagenically equivalent' when they produced the same frequency of lethals in the same type of cell. This method was used also for comparing doses of mustard gas with X-ray doses; a mustard gas exposure, for example, which produced 6% sex-linked lethals, was considered equivalent to an X-ray dose of about 2000 r. Naturally, this estimate of dose can be applied only to effects other than sex-linked lethals themselves; in Auerbach & Robson's work it was used, for example, in determining the relative efficiency of X-rays and mustard gas in producing translocations and large deletions. Apart from this limitation, the method has obvious disadvantages, as the authors point out themselves. The necessity of running a statistically adequate parallel test for sex-linked lethals increases the work considerably. A more serious objection is that to use induced lethals as an estimate of dosage implies part of what is to be proved or disproved by the experiments, namely the essential similarity between X-ray lethals and chemical lethals, and also between lethals produced by different chemicals or by different doses of the same chemical. Nevertheless, for the time being this method seems to be the best available for *Drosophila* work. It certainly is preferable to simple determination of the concentration in the outside medium, and it also is superior to dosage estimates based on toxicity for the exposed flies. These may be very misleading, for where the toxic effect is distinct from the mutagenic one, the two may have quite different thresholds and curves of sensitivity.

Darlington & Koller (1947), in their work on *Tradescantia*, have measured the dose of mustard gas which reached the nucleus in terms of frequency of chromosome breaks, and on this basis have drawn quantitative comparisons with nuclear effects of X-rays.

Timoféeff's second requirement, that of adequate controls, acquires particular significance in the search for weak mutagens whose effects may hardly exceed the range of normally occurring fluctuations of spontaneous mutability. In work of this type particular importance must be attached to the control of incidental conditions which may influence spontaneous mutability. How far such a control is possible depends on our knowledge of the sources of variation in spontaneous mutation rates. We know that in *Drosophila* the age of the fly, the time during which the sperm was stored in the male or in the seminal receptacles of the female, as well as the presence of genes influencing mutability are among the responsible factors; they ought to be kept as strictly similar as possible in treated and control series. With other organisms we are much more in the dark about spontaneous mutability, and not much can be done to reduce its uncontrolled fluctuations. On the statistical side the validity of the conclusions is reduced when one set of controls is used for a number of different experiments, as appears to have been done in much of the older work. The most reliable estimates of the effects of weak mutagens are obtained when the same sub-

stance is tested in replications, each with its simultaneous controls. This makes it possible to estimate the variation within controls, the variation between controls and treated series, and finally the residual variation of the experiment. Data from experiments with weak mutagens, in which these precautions could not be used, are better regarded with reserve.

III. CHOICE OF MATERIAL. SPECIAL METHODS

Drosophila still remains the best subject for the analysis of mutagenic effects. Special methods, most of which have been worked out by Muller, allow of an accurate quantitative determination of the number of mutations in a tested sample of germ cells. These methods are described in many text-books of genetics; the two most important ones are included in Timoféeff's review (1934). By far the most suitable method for preliminary trials with a new substance is a test for sex-linked lethals, by the *ClB* method, or some equivalent. Essentially it consists in the treatment of mature spermatozoa inside the body of adult males; the daughters, which, of course, are heterozygous for one set of treated chromosomes, are bred to produce an F_2. Each F_2 culture represents one treated spermatozoon, and absence of males with the treated *X*-chromosome indicates that a sex-linked lethal had arisen in the corresponding spermatozoon. This method allows the study of fairly large samples without excessive labour, and it eliminates the personal error which is unavoidable in the scoring of visible abnormalities. It loses in statistical accuracy when pre-imaginal stages are treated, or when treated adult males are used long enough to allow maturing of spermatozoa which at the time of treatment had not yet entered meiosis. In these cases the F_2 cultures do no longer represent an equivalent number of tested germ cells, but an unknown smaller number of ancestral germ cells, and although this does not affect the value of the mutation rate itself, it increases the attached sampling error (Auerbach, 1943; Fano, 1943).

There is also another reason why adult insects appear particularly suitable for the detection of chemical mutagens. So far, all agents of high mutagenic efficiency have also been found to break the chromosomes and to interfere with mitosis. Their toxic action is thus directed preferentially against dividing cells. An adult insect, in which hardly any cell division takes place outside the germ line, may of course be highly sensitive to the general toxic effects of a tested substance; but it will at least be able to withstand the very same effects which are to be tested in the germ cells. In agreement with this, it has been found (Auerbach & Robson, 1947*a*) that treatment of adult *Drosophila* males with mustard gas readily produces high mutation rates at doses which do not seem harmful to the exposed flies themselves, whereas developmental stages (embryos, larvae, pupae) show high mortality even at slightly mutagenic doses.

In general, penetration to the germ cells of *Drosophila* offers difficulties. Various methods have been tried: bathing of eggs with and without their chorion; insertion of crystals into the abdominal cavity of imagines; injection into larvae; injection into

imagines; addition of powdered or dissolved substances to the food of the larvae exposure of eggs, larvae or imagines to vapours or aerosols; immersion of explante ovaries followed by re-implantation into fresh larval hosts; treatment of sperm i the vagina of the female. With the exception of the first three methods, clear positiv results have been obtained with each. The suitability of one or the other of ther depends on the substance used, its solubility, its power of penetration, its toxicity and the chemical reaction which leads to mutation. Thus injection into imagine proved satisfactory for urethane (Vogt, 1948), but unsatisfactory for substances c high general toxicity such as nitrogen mustard or sodium desoxycholate (unpublished) On the other hand, these latter substances yielded positive results when applied a aerosols to imagines (Auerbach & Robin, 1947*b*; Demerec, Witkin, Newcombe & Beale, 1947; Demerec, 1947*a*). The aerosol method also gave positive results fo carcinogens (Demerec, 1947*a*, *b*) which had appeared completely negative whe tested by injection (Auerbach, 1940) or by feeding (Bhattacharya, 1948). I this case, however, the superiority of the aerosol method may have been spurious the mutagenic efficiency of carcinogens in *Drosophila* is so low, even when they ar tested by the aerosol technique, that the numbers used by Auerbach and by Bhatta charya were not big enough to make their negative results statistically different fror the positive ones which Demerec obtained in very much larger tests. That eve small differences in technique may influence the result markedly is shown by th fact that Auerbach & Robson (1947*b*), using a rather crude, but only mildly toxi aerosol method, obtained from 5 to 13% sex-linked lethals with nitrogen mustard whereas Demerec (1947*a*), with a more elaborate, but apparently more toxic aeros technique, obtained only 1·7%.

The success of the aerosol method depends also on the type of cell studie Whilst the method proved excellent for inducing mutations in the germ cells c adult males by mustards, it was much less satisfactory for the treatment of female (unpubl.). This might be due to a difference in air supply between ovaries and testes more probably, however, it is caused partly by differential sensitivity of male an female germ cells (see § IV, 2 (*a*)), and partly by the greater protection afforded t the chromosomes of the oocyte by nurse cells, yolk and cytoplasm.

Protection of the chromosomes against chemical influences is probably als responsible for the generally poor results when young embryos are treated. The onl instance in which treatment of fertilized eggs led to very conspicuous genetical effect is the high frequency of somatic mosaics which Auerbach (1945) obtained b exposure of heterozygous embryos to mustard gas vapours; but in this case a exceptionally penetrating vapour was used. Moreover, the chromosomes under tes were those of the superficially situated somatic cells of the embryo. The embryoni germ cells, which in the vast majority of mutation experiments are the object of th test, are retracted into the interior at an early stage of development, and therefor only exposure during a very short period of embryonic life gives some promise c penetration for all but the most powerful substances. Bathing of young egg whether with or without chorion, has so far not yielded a really clear effect with an

of the chemicals tried. It is doubtful whether the chorion offers much resistance to penetration; but that the vitelline membrane is impermeable even to molecules of moderate size was shown in unpublished tests by Callan & Auerbach in which dechorionated eggs were kept for several hours in $2{\cdot}5 \times 10^{-5}$ and $2{\cdot}5 \times 10^{-4}$ M solutions of dinitrophenol; the complete lack of effect on metabolic and developmental rate showed that no penetration had taken place. Other substances which are able to penetrate into the egg may yet fail to produce genic effects because they kill the embryo through their action on the cytoplasm. This may account for the failure of Niggli (1948) to obtain mutations by immersion of eggs in a 20% solution of phenol, although immersion of larval ovaries into a 0·1% solution gave striking mutation frequencies.

This beautiful method of treating explanted larval ovaries has so far only been used by its originator, Hadorn, and his pupil, Niggli. Its two obvious advantages are ease of penetration and avoidance of general toxic effects. Both these advantages might be gained to an even higher degree if it were possible to treat sperm *in vitro*; but this has to wait until a satisfactory technique for artificial insemination in *Drosophila* has been developed. Meanwhile, Herskowitz (1947) has designed a substitute method which consists in flooding the vagina of the female with the substance shortly before copulation. In a small-scale trial with one of the nitrogen mustards, the effectiveness of the method could be demonstrated. It is, however, very doubtful whether it will be suitable also for less penetrating and potent substances, i.e. for just those substances for which it is designed; for according to Herskowitz's observations the injected fluid does not appear to penetrate into the seminal receptacles of the female, so that contact with the sperm can only be very short-lived. As a matter of fact, his success with nitrogen mustard, although undoubtedly real, is quantitatively not impressive. A 10% solution produced a hardly better effect than did a 0·1% solution injected into adult males (unpubl.). Only if means could be found to introduce the injected fluid into the seminal receptacles could this technique develop its full value.

The feeding method has perhaps been used more often than any of the others, although for reasons stated in § II it is the least reliable one. It has, however, led to a spectacular success in Rapoport's (1946) work with formalin and in repetitions of his experiment by Kaplan (1948) and Auerbach (unpubl.).

There are good reasons for wishing to extend work with chemical mutagens to other organisms than *Drosophila*. Obviously, one is not justified in attributing to a chemical mutagen the same lack of organism-specificity which is found in high-energy radiation. Substances which have proved effective mutagens in *Drosophila* cannot, without trial, be assumed to have the same ability in, say, mammals or birds. Such trials may not only be interesting from a theoretical point of view; they will be the necessary preliminary to any type of applied mutation work. Conversely, substances which give negative results with *Drosophila* may yet be mutagenic in a different physiological and genetical environment. This, as Carr (1947) has pointed out, may, for example, be true of carcinogens; their inability to produce tumors in cold-

blooded animals might mean that the really effective agent is a metabolite, produced from carcinogens in the metabolism of warm-blooded animals, but not, or only in small quantities, in cold-blooded ones. In this case, tests for mutagenic potentialities of carcinogens would have more hope of success in the mouse than in *Drosophila*.

So far, the only experiments on chemical mutation in which animals other than *Drosophila* have been used are those by Strong (1945*a*, *b*, 1946*a*, 1947, 1949) and by Carr (1947*b*) on the action of carcinogens on mice. Since these experiments were primarily concerned with the production of cancer, the carcinogens were applied as subcutaneous injections. Different methods, which would ensure better contact of the substance tested with the germ cells, might be more suitable for mutation work proper. Such methods are now being tried out in Edinburgh with a nitrogen mustard.

Unfortunately, the mouse is a very unsuitable object for accurate quantitative mutation work. Dominant visible mutations are much too rare to allow of a comparison between frequencies obtained in treated and control series, and the detection of recessive visibles is limited by requirements of space, cost and labour. Finally, the detection of visible mutations is complicated by personal error, and their testing by incomplete manifestation of many among them. These last two difficulties could be avoided if, by radiation or otherwise, a strain carrying a chromosome with an inverted segment could be established. This would allow accurate scoring of every lethal in either the inverted segment or the corresponding one on the homologous chromosome. In addition, the scope of work would be considerably extended; for lethals are presumably more readily induced than visible mutations, and the crosses carried out for the detection of lethals would incidentally reveal the presence of recessive visibles as well. Another means of extending the field of detection of visible recessives would be the inclusion of less obvious phenotypical characters such as abnormalities of internal anatomy and physiology, blood groups, and factors for tumour tolerance. It may also be possible to induce somatic mutations (including chromosome loss and deletions) by treatment of multiply heterozygous embryos, a technique which would allow scoring in F_1. The only type of genetical change which in radiation work with mice has been amenable to quantitative treatment, is the production of translocations resulting in semi-sterility of the heterozygote (Snell, 1935; Hertwig, 1940). But even here, only a very potent mutagen or one specifically inclined to produce rearrangements rather than gene mutations would yield statistically useful results in experiments of moderate scope.

Although *Drosophila* can be used with advantage also for the detection of chromosomal effects such as chromosome loss, non-disjunction, fragmentation, and rearrangements, it is inferior in this respect to plant material. Detection of chromosomal abnormalities in *Drosophila* is always separated from the moment of treatment by at least several cell divisions, and therefore is necessarily always carried out on a highly selected sample. In plants, on the other hand, chromosomal abnormalities can be detected in the treated cells themselves, and complete scoring is possible. Various methods of chemical treatment have been applied: exposure of flower buds to vapours or aerosols (Koller, Ansari & Robson, 1943; Darlington & Koller, 1947),

bathing of seeds (Stubbe, 1940; Gustafsson & MacKey, 1948), of root tips (Levan & Hin Tjio 1948*a*, *b*; Ford, 1949), and of cut inflorescences (Oehlkers, 1943, 1946) in a fluid containing the substance to be tested. The precautions to be taken in respect of penetration, use of controls, etc., are similar to those discussed for *Drosophila*. They are dealt with specifically in the quoted papers. One point cannot be over-emphasized: the impossibility of comparing results obtained in different organisms and on different types of cell. Differences in permeability, sensitivity, metabolic and other physiological processes have such a large share in the final result that it would be meaningless to compare, for example, mutation rate in *Drosophila* sperm with break frequency in *Allium* roots. When different types of genetical effect are to be compared, it is imperative that this should be done on the same type of cell, if possible in the same experiment. Comparisons of this kind have been carried out for spontaneous and induced mutations in maize. Recently, Lewis (1948, 1949) has developed a technique for detecting sterility mutations in plants which, like the lethal technique in *Drosophila*, is strictly objective and accurate and may make it possible to use plant material for a quantitative study of both mutations and chromosome aberrations.

Micro-organisms have great advantages for chemical mutation work. Penetration is easy, speed of reproduction high, and relatively enormous numbers can be worked with. Techniques have been developed which permit the isolation of mutants as the only survivors in an environment which is lethal to the non-mutated parent strain (radiation, poisons, bacteriophages, antibiotics). These methods resemble the *ClB* test of the *Drosophila* workers in accuracy and objectivity. In certain fungi with sexual reproduction, like *Neurospora* and yeast, Mendelian tests of the usual kind can be carried out to verify the chromosomal basis of the mutations. With the aid of an elegant method devised by Pontecorvo (1949) this can be done even in homothallic species like *Aspergillus nidulans*, in which the multinucleate nature of the hyphae cells combined with self-fertility makes controlled matings impossible. Finally, in fungi without sexual phase like *Penicillium*, an analysis of heterocaryosis may to a certain extent replace that of heterozygosity. All the same, the genetics of even *Neurospora* and yeast is so much less well explored than that of *Drosophila* that for a more detailed analysis of mutagenic effects *Drosophila* still remains indispensable.

Those general features which make micro-organisms so suitable for work with chemical substances are possessed to the highest degree by bacteria. Since Lederberg (1947) has shown that at least one strain of *Escherichia coli* possesses factors which, like the genes of higher organisms, are capable of recombination, it would seem that bacteria are an ideal material for chemical mutation work. It has to be kept in mind, however, that, so far as I am aware, no attempts have yet been made to determine the genetical nature of chemically obtained hereditary aberrations in bacteria; in fact all these aberrations appear to have been obtained in a strain of *Escherichia* which in Lederberg's experiment failed to exhibit characteristic gene behaviour. No doubt, it is only a question of time until these two lines of bacterial research will be combined; but until then—unless one wants to give up the definition of mutations as changes in

chromosomes or genes—it would seem wiser to reserve judgement as to the nature of chemically induced 'mutations' in bacteria. The experience of Ephrussi (1949), who with acriflavine treatment of yeast obtained specific hereditary aberrations which yet probably are not gene determined, illustrates the type of complication which may arise in work with micro-organisms.

IV. RESULTS

1. *Vesicants and lachrymators*

The first substance to show definite and even spectacular evidence of mutagenic ability was mustard gas. The idea to test this substance was conceived at the beginning of the second world war by Dr J. M. Robson, who at that time was studying the pharmacology of war gases. The striking similarity between mustard gas burns and X-ray burns, together with the observation that mustard gas inhibits mitosis in hormonally stimulated vaginal tissue made him consider the possibility that, like X-rays, it might act specifically on the chromosomes. This possibility was tested in collaboration with myself.

The gas was applied to *Drosophila* adults as vapour or aerosol (for details of technique see Auerbach & Robson, 1947*a*; Carr, 1947*a*). Preliminary experiments (Auerbach & Robson, 1942*a*, *b*, 1947*a*) showed that both sexes are wholly or partially sterilized by non-lethal doses, partly through interference with gametogenesis, partly through embryonic lethality after treatment of either parent. The first test for sex-linked lethals was carried out in 1941 (Auerbach & Robson, 1942*a*, 1946) and established at once the correctness of Dr Robson's hypothesis; 7% lethals were obtained in about 1300 treated chromosomes, as against 0·2% in about the same number of control chromosomes. Some of the lethals were connected with large rearrangements, and many with small deficiencies (Slizynska & Slizynski, 1947). Subsequent tests fully confirmed this first finding and yielded even higher mutation rates (cp. Table 1).

Table 1. *Chemical formulae and efficiency of mutagens*

Formula	Designation*	No. of tests	% sex-linked lethals	
			Treated	Controls
A. Mustard group:				
$(ClCH_2.CH_2)_2S$	Mustard gas	Many	Up to 25	0·2
$O(CH_2.CH_2.S.CH_2.CH_2Cl)_2$	Sulphur mustard C	1	8·5	—
$CH_3.N(CH_2.CH_2Cl)_2$	Nitrogen mustard A	3	5–13	—
$N(CH_2.CH_2Cl)_3$	Nitrogen mustard B	1	6·2	—
B. Lachrymators:				
$CH_2:CHNCS$	Mustard oil, allyl *iso*-thiocyanate	2	2·2	0·4
$ClCH_2.CO.CH_3$	Chloracetone	5	Up to 3·3	0·1
$ClCH_2.COCH_2Cl$	Dichloracetone	1	0·8	—

* For the purpose of this article, the various mustards have been designated by letters.

Soon after that, Auerbach & Robson (1947*b*) found that a number of chemically and pharmacologically related substances produced similar effects. Their formulae

and mutagenic efficiencies are shown in the first part of Table 1. Chemically, the mustards consist of chloro-ethyl chains which are attached to a central sulphur or nitrogen atom. According to the nature of this atom, sulphur and nitrogen mustards are distinguished. There can be no doubt about the efficiency of the mustards as mutagens. Confirmatory evidence has been produced on *Drosophila* by Demerec (1947*a*) and Herskowits (1947); on barley by Gustafsson & MacKey (1948); on *Neurospora* by Horowitz, Houlahan, Hungate & Wright (1946), Tatum (1946, quoted in Bonner, 1947) McElroy, Cushing & Miller (1947), and Giles (1948); on *Penicillium* by Stahmann & Staufer (1947); on *Coprinus* and *Ophiostoma* by Fries (1948*a*, *b*); on *Aspergillus* by Hockenhull (1948); on bacteria by Tatum (1946) and Bryson (1947, 1948). Experiments on mice, using nitrogen mustard, have just been started by myself in collaboration with Dr D. S. Falconer (Auerbach & Falconer, 1949) and have so far proved encouraging; one visible recessive mutation was found in a sample of sixteen treated gametes, not all of which had been adequately tested. The ability of the mustards to produce breaks and rearrangements in plant chromosomes was demonstrated for *Tradescantia* by Koller *et al.* (1943) and by Darlington & Koller (1947), for *Vicia* by Ford (1949), for *Allium Cepa* by Steinegger & Levan (quoted in Levan & Hin Tjio, 1948*b*). The powerful action of the mustards on mitosis was detected independently during the war (for references see Gilman & Philips, 1946), and has since been made use of in the treatment of leukaemia and Hodgkin's disease with nitrogen mustard.

The second part of Table 1 presents results obtained by Auerbach & Robson (1944, 1947*b*; also Auerbach, Ansari & Robson, 1943) in experiments in which *Drosophila* males were exposed to vapours or aerosols of a number of lachrymators. Of these, allyl *iso*thiocyanate, which in two experiments gave the same lethal rate of 2·2%, is an undoubted mutagen, although much less potent than any of the tested mustards. It also appears to act as a weak mutagen on *Ophiostoma* (Fries, 1948*b*). It is interesting that a related compound, potassium cyanate, has been reported to give a significant increase in the rate of visible mutations in *Antirrhinum* (Stubbe, 1940). Whether or not allyl *iso*thiocyanate produces also chromosomal aberrations has not been established. One large deletion was found in a test involving several thousand progeny. Since it seems possible that also after chemical treatment, as after X-radiation, the frequency of large rearrangements follows approximately the square of the dose, while that of lethals is directly proportional to dose, much larger tests would be required to prove the production of rearrangements by such a weak mutagen as allyl *iso*thiocyanate.

Chloracetone produced varying results in five experiments. In the only one which was done with simultaneous controls, the difference between mutation rate in the treated series (1%) and in the controls (0·1%) was on the borderline of statistical significance ($P=0{\cdot}05$). The same is true of the comparison between this set of controls and the pooled data for both chloracetone and dichloracetone; but for reasons discussed in § II such a comparison is of doubtful validity. On the whole, it seems probable that both chloracetones are weak mutagens.

Not all vesicants and lachrymators, however, even when highly toxic, are mutagens in *Drosophila*. Decidedly negative results were given by tests with two potent vesicants: lewisite (Cl_2: As . CH : CHCl) (Auerbach & Robson 1947*b*) and chloropicrin (unpubl.). Negative results were also obtained with osmic acid vapour and picric acid, which were tested because they resemble mustard gas in being fixatives of protoplasm (Auerbach & Robson, 1947*b*). The criticism which can be directed against inferences from negative data in general, and especially when they have been obtained with sublethal doses, has been discussed above and applies also to these experiments.

2. *Analysis of mustard effects*

The discovery of a new mutagenic agency opens up two main lines of research. First, the testing of related agencies may reveal a more general mutagenic principle which is common to them all; secondly, closer analysis of the action of one representative mutagen of the group is likely to contribute to our understanding of the mechanism of mutation in general. Thus, the discovery of the mutagenic action of X-rays was followed on the one hand by experiments with other types of radiation and by the realization that all ionizing radiations resemble X-rays in mutagenic efficiency; on the other hand, by a detailed study of X-ray effects which more than any other line of research has increased our knowledge and clarified our conceptions of mutation. Research on chemical mutagens has so far done only the first steps in either direction. The previous paragraph has presented data from experiments in which substances of chemical or pharmacological resemblance to mustard gas were tried out. Data on other chemical substances, which have been tested independently, will be reported below. The only chemical mutagen which so far has been subjected to a closer study of its action is mustard gas. A brief review of the results of this analysis will be presented before going on to other substances. (See also the summaries presented by Auerbach, Robson & Carr, 1947, and by Auerbach, 1949.)

The main emphasis in this analysis has been laid on possible differences from X-ray action; for a detection of differences between the mechanism of physical and chemical mutagenesis seems a promising approach to the problem of the mechanism of mutation in general. From this point of view it may seem disappointing that so far only a few differences have been found, the main ones being a relative shortage of gross chromosomal rearrangements and the occurrence of delayed mutation after mustard gas treatment.

(*a*) *Lethals in* Drosophila

Sex-linked lethals were found to be distributed along the whole of the *X*-chromosome in a similar manner as are lethals induced by X-rays (Slizynska & Slizynski, 1947). Autosomal lethals in the second chromosome were between 4 and 5 times as frequent as sex-linked lethals in an experiment in which well over 1000 chromosomes of either type, both derived from the same treated males, were compared (Auerbach, 1949). This ratio is considerably higher than would be expected from a comparison of the physical lengths of the two chromosomes. It also appears to exceed the ratio

between autosomal and sex-linked lethals after X-radiation; but the data are not sufficient to establish this point.

The frequency of induced sex-linked lethals varies with the stage of the germ cell at the time of treatment (Auerbach, 1949). Whereas X-rays produce their highest effects on mature spermatozoa, mustard gas acts most readily on somewhat younger stages. This was shown in experiments in which treated males were given fresh females every few days so that sperm could be used continously as it kept on maturing. In five out of six experiments the frequency of sex-linked lethals reached a peak in spermatozoa which were used about 6 days after treatment.

The production of lethals in female germ cells of *Drosophila* is difficult (unpubl.). In three experiments in which males and females were exposed together, the frequency of sex-linked lethals in mature sperm was 8–15 times that in mature ova. In one of these experiments the treated females were kept for about a month and frequently changed to fresh culture bottles in order to test ova which at the time of treatment had still been in the terminal chamber of the ovariole, in which the chromosomes are not yet shielded by yolk, large amounts of cytoplasm, and nurse cells. The fact that the initial low mutation frequency remained unchanged during the whole period suggests that the sex difference in response to treatment is due not only to the better protection of the ovarian chromosomes, but also either to a real difference in sensitivity of the chromosomes in male and female germ cells, or to some secondary process—e.g. 'recovery' from the initial effects, or selection for survival of gametes with unaffected chromosomes—which occurs only or preferentially in female germ cells.

No increase of mutation rate was found in untreated *X*-chromosomes which through fertilization had been introduced into treated ova, although in these tests the maternal chromosomes showed a definite, if slight, effect of the treatment (Auerbach & Robson, 1947*a*; also Auerbach, unpubl.). This indicates that treated cytoplasm does not in its turn become mutagenic.

(*b*) *Centromere effect*

Darlington & Koller (1947) found that one effect of mustard gas on *Tradescantia* consisted in a weakening of the centromere which later could lead to breakage and misdivision. Auerbach (1947*a*), in the progeny of treated *Drosophila* males, observed several abnormal types of segregation which point to the existence of a centromere effect also in this material. Only one of these cases could be analysed completely; its most plausible interpretation was that the treatment had somehow 'weakened' the centromere of the *X*-chromosome, and that this had resulted in a tendency of the treated *X* to follow its homologue either into the sister cell or half-way along the spindle with subsequent loss.

(*c*) *Structural changes in plant chromosomes*

Koller *et al.* (1943) and Darlington & Koller (1947) have studied the effects of the mustards, mainly of mustard gas itself, on mitosis and meiosis in *Tradescantia*. They

found three main effects of the treatment: (*a*) the centromere effect which has been mentioned above, (*b*) stickiness and abnormal spiralization of the chromosomes, and (*c*) breakage of the gene string which results in fragments as well as new types of reunion. According to the latter authors, the effects of mustard gas are closely similar to those of X-rays of low intensity. Ford, however, who studied the effects of nitrogen mustard on *Vicia* root tips, obtained evidence which suggests that in his material the distribution of the primary breaks differs after the two types of treatment (1949; also personal communication).

(*d*) *Structural changes in* Drosophila

Slizynska & Slizynski (1947) carried out a cytological examination of eighty-nine sex-linked lethals which had been produced by mustard gas; they all came from the same experiment in which the treatment had induced 7% sex-linked lethals. The sample contained about 20% small deficiencies, a figure which is very close to similar ones obtained by the same authors on X-ray lethals and ultra-violet lethals. On the other hand, only six out of the eighty-nine lethals were connected with large rearrangements. This is considerably less than would be expected in a similar X-ray sample; for lethals produced by a mutagenically equivalent dose of X-rays (see § II) include about 30% with large rearrangements. This relative shortage of large rearrangements was confirmed by genetical tests for the frequency of large deletions and translocations in the progeny of mustard gas-treated males (Auerbach & Robson, 1947*a*). Table 2 summarizes the results. In this table the expected frequency of

Table 2. *Frequency of large rearrangements in the progeny of mustard gas-treated males*

Experiment	Percentage sex-linked lethals	No. of tested spermatozoa	Translocations between autosomes II and III		
			Observed	Expected	Ratio
H32	8·6	816	7	56	1:8
H89	14·5	981	21	98	1:5
		1797	28	154	1:6
			Large deletions in the *X*-chromosome		
H92	9·1	6635	16	41	1:2·5
HX1	6·8	5052	9	18	1:2
		11687	25	59	1:2

rearrangements is that which a mutagenically equivalent dose of X-rays would have produced in a sample of the same size. Both types of rearrangement fall short of expectation, translocations significantly more so than deletions ($\chi^2 = 10$ for 1 D.F.). This latter finding suggests that the shortage may be more pronounced for inter-chromosomal than for intrachromosomal rearrangements.

(*e*) *Somatic crossing-over in* Drosophila

Treatment of embryos heterozygous for three sex-linked marker genes led to the appearance of a very high number of spots and twin spots in the imagines (Auerbach, 1945). Most probably all of these spots were caused by somatic crossing-over. The spots tended to be very small, markedly smaller than after irradiation of embryos of similar age (Patterson, 1929). This indicates that crossing-over had not always occurred in the treated cells themselves, but at least sometimes later in cells derived from the originally treated ones. Since we do not know how long mustard gas or an active derivative of it can persist in the embryo, it is not possible to decide whether this observation indicates delayed effect on the chromosomes (see below), or whether it simply is due to the active principle still being present at a later developmental stage.

(*f*) *Visible mutations*

In *Drosophila* the proportion of visible to lethal mutations appears to be approximately the same after treatment with mustard gas as after irradiation (Auerbach & Robson, 1947*a*). No specific effects of the treatment on individual genes was observed. In barley, Gustafsson & MacKey (1948) found that nitrogen mustard, applied in solution to dormant seeds, results in a non-random production of mutations, certain albino types being more, and others less, frequent than after irradiation. The results with *Drosophila* and barley are not necessarily contradictory; as the Swedish authors point out, the work of Auerbach & Robson has shown specificity of mustard action in the sense that certain types of structural change are less frequent than after mutagenically equivalent doses of irradiation. Until the exact structural nature of the various albino mutations is known, it therefore remains possible that also in barley the specificity of mustard action refers to types of structural change rather than to mutation at specific loci.

(*g*) *Mosaics in* Drosophila

One peculiarity of visible mutation after mustard treatment of *Drosophila* males is the frequent occurrence of mosaics in their progeny (Auerbach, 1946). These form about 50% of all visible mustard mutants, as compared with 10–15% of X-ray mutants. Mosaics in the progeny of X-rayed males are presumed to arise at the first cleavage division. According to Patterson (1933) they owe their origin to the precocious splitting of a certain proportion of sperm chromosomes into sister chromatids. According to Muller (1940), on the other hand, all sperm chromosomes are still unsplit, but breaks which have been induced in them may remain latent until splitting has occurred in the fertilized ovum; differential reunion in sister chromatids may then result in mosaicism. That this latter mechanism may be responsible for certain mustard mosaics was shown by a mosaic which in one part of its body was deficient for the same chromosome segment which was duplicated in the other (unpubl.).

It is, however, not possible to explain all mustard mosaics in either Muller's or

Patterson's way; for there is evidence that they can arise much later than at the first cleavage division. This evidence was obtained in several independent lines of research (Auerbach, 1946, 1947*b*). First, it was found that the size of the mosaic area in mustard mutants usually covers less than half the body surface. Next, mosaicism involving the gonads, which is rare after X-radiation, is frequent after mustard treatment. This was shown by an analysis of apparent semi-lethals, i.e. of daughters of treated males which gave a low proportion of sons with the treated *X*-chromosome. It was found that about half of these females were in reality gonadic mosaics for a full lethal; i.e. they carried a lethal in part only of their gonads, its normal allelomorph in the remainder. The best evidence for delayed occurrence of a mutation after mustard treatment was the finding that among the progeny of a number of such gonadic mosaics there were again gonadic mosaics for the same mutation. In these cases, a whole generation must have elapsed between treatment and mutation. The fact that mosaics were found also in the progeny of mustard-treated females is further confirmation of a delayed effect; for the chromosomes of the unfertilized ovum have still to undergo the meiotic divisions before entering upon the cleavage divisions, and a mutation induced at the time of treatment, even if it involved only one chromatid, would not result in mosaicism.

The most plausible, although not the only possible, interpretation of these observations is the assumption that chemical treatment, in contrast to X-radiation, may induce a labile premutation which subsequently, after one or more cell divisions, may give rise to the actual mutation or may revert to the old allelomorph. The primary instability may or may not have its own phenotypical expression. As an example of the first possibility a mutation to rudimentary may be cited. It arose first as a mosaic; in some of the lines derived from this mosaic the mutation proved unstable and reverted to wild type, in others it remained stable (unpubl.). A primary instability without phenotypical expression seems to have been present in two triple mosaics which in addition to two genotypically and phenotypically different rearrangements involving the same locus contained a phenotypically wild-type area (unpubl.).

3. *Phenols*

In 1940 Stubbe reported the results of experiments in which seeds of *Antirrhinum* had been exposed to solutions of various chemical substances and the F_2 examined for mutations. A significant increase in mutation rate was found for potassium thiocyanate; this has been mentioned above (§ IV, 1). Two other substances, chloral hydrate and phenol, gave at least very suggestive results, the difference from the controls being more than twice its standard error.

Meanwhile, phenol has been applied to *Drosophila melanogaster* by Hadorn and his collaborators, using their novel method of treating explanted ovaries (Hadorn & Niggli, 1946; Niggli, 1948; Hadorn, Rosin & Bertani, 1949). Their results are very striking, and in some respects still unexplained. There can be no doubt about the effectiveness of their treatment. Only second-chromosome mutations were scored. Of 241 treated ovaries 44 (= 18·3 %) had one or more mutations.

Altogether 142 mutations were obtained in 2519 treated chromosomes (88 full lethals, 21 semi-lethals, 33 visibles). No mutation was found in 508 chromosomes from ovaries which had been transplanted without chemical treatment, and only 1 lethal in 772 completely untreated control chromosomes. Two features of this work remain unexplained. The first is its failure to give repeatable results; although positive results of high significance (up to 19·5% lethals) were obtained in many series, others carried out in exactly the same way remained completely negative. The authors have not yet been able to decide whether this is due to the active agent being a contaminant of phenol rather than phenol itself, or to phenol being able to act as a mutagen only under very special physico-chemical conditions which happened to be fulfilled in the positive experiments.

The second, even more intriguing, problem is that of the specificity of the treatment which seems to manifest itself on three levels: as chromosome specificity, strain specificity, and locus specificity. Chromosome specificity is suggested by Niggli's observation that the sex ratio among the F_1 from successfully treated ovaries was if anything higher than normal. If all chromosomes responded equally to the treatment, one would expect that phenol, which in these experiments produced about 16% lethals on the second chromosome, would kill a proportion of F_1 males through sex-linked lethals. However, this question can only be decided by special tests for sex-linked lethals. So far, *ClB* tests on males which had been injected with phenol gave negative results; but since it is not known whether injection allows penetration of phenol to the germ cells, no conclusions can be drawn from these data. Much better and very startling evidence is available for locus and strain specificity of the response to phenol. Tests for allelomorphism were carried out on samples of lethals and showed, as would be expected from any effective treatment of immature ovaries, that the same lethal tended to appear repeatedly in the progeny of the same treated female. But, in addition, there was found an unexpected and remarkable degree of interovarial identity of lethals, even between ovaries belonging to different experimental series, separated from one another by more than two years. On the other hand, no identical lethals were found among samples obtained in two different wild-type strains. Niggli interprets these facts as suggesting the existence of specific 'phenol-sensitive' loci which differ between strains. If such loci did, in fact, exist, it seems likely that their sensitivity to mutagenic influences would not be restricted to phenol. In particular, one would expect some of the repeatedly induced lethals to be present in the untreated strain as a result of 'spontaneous' mutation. It would be interesting to test Niggli's hypothesis by analysing, for each of her tested strains, naturally occurring lethals as well as lethals induced by some other weak mutagen.

Levan & Tjio (1948*a*, *b*) have tested the effects of some forty phenols, phenol derivatives and related compounds on the chromosomes of *Allium* roots. In certain concentrations, which were lower than the markedly toxic ones, almost all these compounds were found to produce fragmentation of chromosomes. Phenol itself had very little effect; but striking results (up to over 60% of examined cells containing chromosome breaks) were induced by some of the higher phenols like pyrogallol and

hydroquinone, by quinones like benzoquinone, and by amines like *p*-phenyldiamine. Translocations were rare, most of the fragments either remaining attached to the chromosome by a thin thread or staying behind on the equator as free chromosome pieces. Tests of some of these highly active substances for mutagenic ability seem very desirable.

4. *Urethane (ethyl carbamate)*

Oehlkers (1943, 1946, 1949) studied the effects of a variety of inorganic and organic substances, used singly and in combination, on meiosis in pollen mother cells of *Oenothera* and some other plants. The substances were added to the water in which the cut inflorescences were kept; in later experiments the method was modified so as to allow the treatment of plants in the field, and the collection of pollen for the production of progeny. Chromosome and chromatid translocations were observed after many of the treatments, both with simple salts of potassium, sodium, calcium and aluminium, as also with organic substances such as ethyl and propylurethane, glucose and saponin. The effect could be greatly increased by using certain combinations of organic and inorganic substances. The most effective combination was ethyl urethane with potassium chloride, which in five series of about 100 examined cells each produced translocation percentages ranging from 7 to 38. The sensitive period appears to be restricted to the resting stage with a possible extension into very early prophase stages. Neither duration of treatment nor quantity of administered substance were correlated with the size of the effect, and even the chemical specificity of the substance or mixture seemed irrelevant within wide limits. On the other hand, there is a definite influence of the cytoplasmic environment, as evidenced by differences between reciprocal hybrids in the frequency of induced aberrations. The author concludes that the only important condition is to create a cellular disturbance at a special sensitive stage in which such disturbances are apt to lead to chromosomal aberrations.

Oehlkers's data suggested that urethane might possess true mutagenic ability. Experiments carried out by Vogt in 1948 brought convincing proof for this possibility. Injection of *Drosophila* males with urethane in isotonic potassium chloride solution resulted in over 3% mutations in three different experiments, as compared with 0·6% in the controls. The treated series also contained several recessive and visible mutations, at least one of which was connected with a deficiency. Urethane is a powerful mitotic poison (Guyer & Claus, 1947). Like the nitrogen mustards it has in recent years been used therapeutically as a means of depressing leucocyte counts in leukemia and Hodgkin's disease (Paterson, Haddow, Thomas & Watkinson, 1946). It seems possible that the mutagenic ability of urethane, in particular its capacity to induce chromosomal aberrations, plays a role in its inhibitory action on mitosis.

A variety of carbamates and thiocarbamates inhibit cell division in plants (Templeman & Sexton, 1945). In Oehlkers's experiments with *Tradescantia* (1943) propylurethane resembled ethylurethane in its capacity to produce translocations. Bryson (1949) reported the induction of phage-resistant mutants in *Escherichia coli* which had been treated with methyl-, ethl-, propyl- or butylcarbamate.

5. *Carcinogens*

The cancer cell appears to arise from the normal one by a sudden change which, once it has arisen, is perpetuated through an unlimited number of cell generations. In these respects the transformation of a normal cell into a malignant one resembles a mutation. The hypothesis that cancer is, in fact, due to somatic mutation is old. According to it, carcinogens act by inducing mutations in somatic cells. Applied to reproductive cells they would be expected to produce germinal mutations. Such a parallelism of carcinogenic and mutagenic ability has been demonstrated for physical carcinogens like X-rays and ultra-violet radiation. The most powerful chemical mutagens, the mustards, have not so far been shown to possess carcinogenic effects; but more extensive tests may well yield a positive result. Urethane, which is an undoubted mutagen, produces tumours, although of an unusual type. Attempts to show that known chemical carcinogens are also mutagens have until recently yielded negative or doubtful results. Lately, however, there have been a number of independent reports on the production of mutations by carcinogens.

Strong (1945*a*, *b*, 1946*a*, *b*, 1947, 1949) experimented with a line of mice (Strong, 1940) in which biological variability had been increased on purpose by hybridization between three inbred strains (*CBA* agouti; *JK* non-agouti, brown, pink, spotted; *N* non-agouti, brown, pink, dilute, spotted). Following this initial hybridization, the mice were inbred, and during many generations treated with subcutaneous injections of methylcholanthrene. In later generations a great number of variants appeared within the line as well as in outcrosses to unrelated inbred mice. Many of these aberrations could be shown to be hereditary. They include such well-known recessives as pink, albino, spotted; dominants such as various spotting effects; reverse mutations such as self out of piebald, and black out of brown (the latter apparent reversal occurred eight times, three times in a single litter from brown by brown parents); new types like yellow scrotal hair, white ears; a whole series of shades intermediate between black and brown and presumed to be alleles at the brown locus; and patterned mice which are interpreted as somatic mosaics between different members of this lastnamed series. Other abnormalities like *situs inversus*, increased body weight, large litter size, increased susceptibility to the tumour-inducing effect of the injections are not all considered as necessarily due to mutations; but the frequent coincidence of several of the physiological changes with coat-colour mutation leads the author to postulate that the treatment may create an 'unstable genetic state'. In comparison, it is stated that only eight germinal mutations were found among the more than 210,000 untreated mice which during 27 years had been bred in the same laboratory. As has been discussed above in § III, a statistical evaluation of mutation rates, defined as the number of mutations per 100 tested gametes, is very difficult in mice. Therefore Strong's estimate of a mutation rate of 1 in 557 in the treated series as compared with 1 in 26,250 in the controls cannot have the same well-defined meaning which such a statement would have when applied to, for example, *Drosophila* or *Neurospora*. It does, however, emphasize the striking

difference between the experimental data and the well-known fact that visible mutations occur very rarely in untreated laboratory mice. A detailed publication of the pedigree of all or at least many of the variants and of the results of the genetical tests which have been carried out with them would greatly help other geneticists to arrive at independent estimates of the quantitative side of the work. So far, only one such genetical case history has appeared (Strong, 1946*b*). Carr (1947*b*) has published data which qualitatively are in agreement with Strong's findings. In a small population of mice which for several generations had been treated with dibenzanthracene he obtained seven striking variants, four of them shown to be hereditary and resembling known colour mutants. Unfortunately, the use of a genetically untested and only very mildly inbred male as founder animal of the line leaves room for the possibility admittedly remote, that the mutations may have been present in the line prior to treatment.

In view of the inherent unsuitability of the mouse for quantitative mutation work, tests of carcinogenic substances on more favourable material appear very desirable. These have been carried out recently by Demerec on *Drosophila* by means of a specially devised aerosol technique (1947*a*, *b*, 1948, 1949; and Demerec *et al.* 1947). Full data have so far been published for dibenzanthracene (1948). Table 3 reproduces those referring to the frequency of sex-linked lethals. The difference

Table 3. *Frequency of sex-linked lethals after treatment of* Drosophila *males with an aerosol of dibenzanthracene (abridged after Demerec)*

Exp. no.	Treatment in hours	No. of chromosomes tested	Lethals	
			No.	%
116	12	662	7	1·06
117	6·5	1,341	8	0·60
118	13·5	1,254	16	1·28
182	120	875	14	1·60
202	215	647	2	0·31
Total treated		4,779	47	0·98
Total controls		10,006	28	0·28

(Range of variation in 10 control tests = 0 − 0·36.)

between the mean mutation rates in tested and control series is statistically significant (4·67 times its standard error). Moreover, in four out of five experiments mutation rate in the treated series was beyond the range of variation in ten control experiments. There can thus be little doubt that dibenzanthracene, under the conditions of these experiments, has acted as a weak mutagen.

A striking feature of this work is the high incidence of rearrangements which seem to have been produced by a mutagenically not very effective substance. Of the lethal-bearing chromosomes, forty-three were examined for the presence of large rearrangements. Two of them were found to contain one inversion, and a third had two independent inversions. No rearrangement was present in nineteen examined control lethals. An incidence of four inversions in forty-three lethals would be high

even after a mutagenically equivalent (see § III) X-ray dose (Demerec, 1937). With mustard gas treatment, such a high incidence of inversions was hardly attained by a dose which induced 7% sex-linked lethals (Slizynska & Slizynski, 1947). Furthermore, the great majority of X-ray-induced inversions which occur on the same chromosome as a lethal have one breakage point at or close to the locus of the lethal (Demerec, 1937). The same applied to three of the inversions obtained after dibenzanthracene treatment (personal communication); but unless the chromosome with two independent inversions also had two independent lethals, one of these two inversions must have arisen independently of the lethal through chance coincidence. The occurrence of at least one such coincidence in a small sample is surprising. It suggests either that this particular inversion may have been present in the stock in low frequency even before treatment, or else that after dibenzanthracene treatment rearrangements are exceptionally frequent and may also have been present in chromosomes without a lethal. This highly interesting possibility requires for its establishment special genetical tests for the induction of viable rearrangements by dibenzanthracene. So far, only one test for translocations between the second and third chromosome could be carried out and gave a promising result: 1 translocation was present in 292 tested spermatozoa. More extensive data, however, are required for deciding this important point.

Proof that one or even a few carcinogens possess weak mutagenic ability is not sufficient to make a good case for an intrinsic connexion between mutation and carcinogenesis. Tests of a great number of chemical substances for mutagenic ability are now being undertaken in various laboratories and are likely to lead to the discovery of some strong and many weak mutagens. The fact that several carcinogens were among the first weak mutagens to be detected may simply be due to their having been among the first substances to be tested. It therefore meant an important step forward, when Demerec (1949; also Demerec *et al.* 1947) put the whole work on a broader basis by trying to establish a correlation between carcinogenic and mutagenic abilities of a large number of chemical substances. All substances were applied to young males of the same wild-type stock of *D. melanogaster*, treatment being given as aerosols in which the flies were kept for from 7 to 200 h. The scale of the experiments was large enough to reveal even small increases in mutation rate. The data are summarized in Table 4, which was presented at the VIIIth International Congress of Genetics and has kindly been put at my disposal.

The majority of the substances were chosen from two groups known to contain carcinogens: the polycyclic hydrocarbons and the azo-compounds. Two other substances, sodium desoxycholate and acriflavine, were also tested, but were not considered in the evaluation of the results. In the last two columns each substance is classified in respect of carcinogenic and mutagenic abilities. A comparison of these two columns is taken to indicate a significant correlation between these two abilities. Indeed, six out of seven carcinogens were classed as mutagens; whereas of nine non-carcinogenic hydrocarbons or azo-compounds only two were classed as definite mutagens and one as doubtful. In judging of the significance of this correlation one

has, however, to keep in mind that it was obtained by a definition of mutagenicity which may not meet with the agreement of every geneticist. In addition, the two 'miscellaneous' substances were disregarded, implying that these produce mutations in some different manner, not connected with carcinogenesis. For the definition of mutagenicity two different criteria have been used, only one or the other of which needs to be met. The first is the orthodox one of a significant increase in frequency of sex-linked lethals over the controls, the chosen level of significance apparently being about 2%. The second is the presence of rearrangements among the lethals. Even a substance which, like 2-amino-5-azotoluene, is definitely non-mutagenic by the first criterion, is yet classed as a mutagen if even one rearrangement is detected among the lethals.

Table 4. *Frequency of sex-linked lethals in the progeny of* Drosophila *males after treatment with aerosols of various chemicals (after Demerec)*

Treatment	No. exps.	No. chromosomes	Lethals				Substance	
			No.	%	Test	C.A.	Carc.	Mut.
Control: Not treated	2	2,510	6	0·24				
,, Sesame oil, aerosol	5	7,496	22	0·29				
Total	7	10,006	28	0·28 ± 0·098	19	0		
Hydrocarbons:								
1, 2, 5, 6-Dibenzanthracene	5	4,779	47	0·98 ± 0·053	43	3	+	+
20-Methylcholanthrene	5	4,671	25	0·54 ± 0·11	22	0	+	+
3, 4-Benzpyrene	5	5,262	26	0·49 ± 0·10	24	1	+	+
1, 2-Benzanthracene	2	2,351	10	0·43 ± 0·14	8	1	−	+
Anthracene	2	3,315	13	0·39 + 0·11	13	0	−	−
Phenanthrene	2	2,353	5	0·21 ± 0·094	4	0	−	−
Pyrene	2	1,172	3	0·26 ± 0·15	3	0	−	−
α-Naphthylamine	3	3,532	7	0·20 ± 0·075	5	0	−	−
β-Naphthylamine	6	5,445	18	0·33 ± 0·078	17	0	+	−
Azo-compounds:								
p-Hydroxyazobenzene	3	3,391	47	1·37 ± 0·20	44	3	+	+
p-Aminoazobenzene	2	1,315	19	1·44 + 0·33	16	0	−	+
p-Dimethylaminoazobenzene	6	4,905	12	0·24 ± 0·070	12	1	+	+
2-Amino-5-azotoluene	3	2,917	6	0·21 ± 0·084	6	1	+	+
Azobenzene	2	1,157	7	0·61 ± 0·23	7	0	−	(+)
Azoxybenzene	3	2,421	8	0·33 ± 0·12	8	0	−	−
p-Diethylaminoazobenzene	2	1,609	2	0·12 ± 0·086	2	0	−	−
Miscellaneous:								
Sodium desoxycholate	2	2,561	29	1·13 ± 0·21	28	0	−	+
Acriflavine	2	1,886	13	0·69 ± 0·19	13	0	−	+

C.A. = chromosome aberrations. Carc. = carcinogenic.
Mut. = mutagenic. (+) = slightly positive.

It is obvious why this criterion has intruded: as in the experiments with dibenzanthracene, discussed above, there is a surprisingly high incidence of rearrangements in chromosomes derived from substances which are only weakly mutagenic or do not seem to produce mutations at all. According to a personal communication by Dr Demerec, none of the rearrangements was present in the untreated strain. It therefore seems possible, as Demerec suggests, that these experiments have revealed the existence of substances whose genetical activity is limited to the production of

chromosome breaks. Results from experiments to test this suggestion will be awaited with great interest; for the detection of substances which can break the chromosomes without causing gene mutations would throw new light on the vexed problem of the relationship between these two effects.

However, until this relationship is better known it seems hazardous to use lethal mutations and rearrangements as alternative criteria for mutagenic ability. If, instead, only the ordinary criterion of increased lethal frequency is used, the correlation between carcinogenicity and mutagenicity becomes less obvious. Values from χ^2 tests of significance on the data of Table 4 are presented in Table 5.

Table 5. *Level of significance of the increases in frequency of sex-linked lethals. Data from Table* 4

Substance	Increase over controls	χ^2	*P*	Carc.
1, 2, 5, 6-Dibenzanthracene	0·70	31·7	<0·001	+
20-Methylcholanthrene	0·26	5·8	Near 0·02	+
3, 4-Benzpyrene	0·21	4·5	Near 0·04	+
p-Hydroxyazobenzene	1·09	36·0	<0·001	+
p-Aminoazobenzene	1·16	38·4	<0·001	−
Azobenzene	0·33	3·6	Near 0·06	−
Sodium desoxycholate	0·85	32·8	<0·001	−
Acriflavine	0·41	7·7	Near 0·006	−

For the remaining three carcinogens and seven non-carcinogens, *P* is > 0·1

For three substances, two carcinogenic and one not, *P* is between 1 and 6%. Considering the sensitivity of mutation rates to genetical influences like that of the gene for sensitivity to chemical mutagens stated to be present in the strain, as well as to physiological influences like the storing of sperm in the males, which must have been unavoidable in exposures lasting up to 200 h., it seems wiser to regard these substances only as possible mutagens in the customary sense of the term. In fact, Demerec himself does so for azobenzene for which no rearrangement served as additional criterion of mutagenic ability. Five substances gave increases which were statistically significant at a lower level than the 1% one. Two of these are carcinogens, three are not. If, following Demerec, we include methylcholanthrene as proven mutagen and exclude sodium desoxycholate and acriflavine as acting in a different way, we are left with four definite mutagens, three of which are carcinogens, while the remaining three carcinogens are not mutagenic in the usual sense. Seven substances are neither mutagens nor carcinogens; but in view of the comparative rarity of carcinogens as well as mutagens this coincidence can hardly be used to improve the correlation.

Not included in Table 4 is a further carcinogen, acetylaminofluorene, which induces a high percentage of tumours in rats fed on a diet containing it. Demerec *et al.* (1947) found it non-mutagenic when applied as aerosol to *Drosophila*. This was perhaps to be expected, since this substance seems to require passage through the intestinal tract for its carcinogenic action. But feeding it to *Drosophila* larvae

was likewise without effect on mutation rate, although many larvae developed melanotic 'tumours' (Bird & Auerbach, unpubl.).

Latarjet (1948), in mutation tests on bacteria, failed to establish a connexion between mutagenic and carcinogenic abilities. In particular, he obtained no increase in mutation rate with methylcholanthrene, although fluorescence tests showed it to have penetrated into the cells.

Tatum (1947) treated *Neurospora* with the water-soluble 20-methylcholanthrene endosuccinic acid and obtained six biochemical mutants out of 3075 treated cultures. No controls appear to have been taken; 1507 untreated cultures, used as controls for another experiment by the same worker, contained one biochemical mutant.

One has to keep in mind, however, that a high positive correlation between mutagenic and carcinogenic abilities is not necessarily expected on the somatic mutation theory of cancer. In the first place, it is unfortunately not possible to test both abilities in the same organism. Penetration to the chromosomes and metabolic fate of the tested substances may well differ so much between mammals on the one hand, *Drosophila* or micro-organisms on the other, that a positive correlation becomes obscured. In this context it is interesting to note that Carr at the London Conference of the British Empire Cancer Campaign and the Genetical Society of Britain in 1948 reported encouraging results from preliminary mutation tests on *Drosophila* which had been treated with mammalian metabolites of carcinogens. The necessity of working on two different organisms makes it also difficult to grade the two effects studied in such a way that comparable scales for a more exact correlation table are obtained. This seems important, because neither mutagenic nor carcinogenic abilities are all-or-nothing characteristics, and no sharp dividing line can be drawn between carcinogens and non-carcinogens, mutagens and non-mutagens. Furthermore, mutation to cancer needs must be of a special type resulting in uncontrolled growth. A powerful mutagen with indiscriminate action on all genes would be expected to produce also this specific type of mutation in significant frequency. A weak general mutagen would do so only if the genes whose mutation give rise to uncontrolled growth are particularly frequent in mammalian chromosomes. Other carcinogens may be supposed to act specifically on these particular genes. It might then be difficult or impossible to detect their mutagenic effects in *Drosophila*, because the sensitive genes are not present or are not being tested for mutations.

Several types of specificity of carcinogenic mutation have been suggested. Darlington (1944, 1949) thinks that carcinogens produce mutations in plasmagenes. Koller (1943) and Caspersson (quoted by Koller) assume preferential action on heterochromatic genes. The first hypothesis is difficult to test with present means. The second lends itself to examination by methods which might conceivably prove a more sensitive indicator of mutagenic ability of carcinogens than the orthodox tests for lethals. One such method consists in the scoring of mutations of the fertility genes on the heterochromatic *Y*-chromosome of *D. melanogaster*. It was applied by myself to flies which had been reared on a culture medium containing acetyl-aminofluorene. The result was negative; but repetition on a larger scale and with a substance

shown to raise the frequency of sex-linked lethals seems desirable. The fact that of the eight dibenzanthracene breaks listed by Demerec, five occur in regions which probably are heterochromatic (Slizynski, 1945) is in itself no proof for a preferential action on the heterochromatin, but is at least not in disagreement with such a hypothesis.

6. *Other substances*

Formalin, when mixed with the food of *Drosophila* in sublethal concentrations, results in a striking increase of mutation rate (Rapoport, 1946; Kaplan, 1948; Auerbach, unpubl.). The frequency of sex-linked lethals was 5·92 in Rapoport's experiments, 5·66 in Kaplan's and 5·0 in Auerbach's. Rapoport also reports similar results from tests with other compounds containing the CO group, and he proposes the hypothesis that the effect is due to interaction of the aldehydes with amino-acids of genes and chromosomes. However, a large-scale experiment by the reviewer (unpubl.) in which adult male and female *Drosophila* were exposed to formalin vapour for varying lengths of time was entirely negative. This makes it appear more likely that formalin acts not directly on the genic material, but indirectly through chemical changes in food constituents.

If this were true, it would bring these experiments into line with the very remarkable ones by Stone *et al.* (Stone, Wyss & Haas, 1947; Wyss *et al.* 1947; Stone, Haas, Bennett Clark & Wyss, 1948; Wyss *et al.* 1948), in which bacterial mutations were induced by treatment of the medium. Originally, the treatment consisted of radiation with ultra-violet light, but this could subsequently be replaced by pre-treatment with hydrogen peroxide. The latest results indicate that the effective substance is some organic peroxide. It appears paradoxical that mutagenic substances should be produced by two compounds, formaldehyde and hydrogen peroxide, which chemically act in opposite ways, the first being a reducing, the second an oxidizing agent; but without further data it would be idle to speculate on this contradiction.

In 1943 Thomas & Chevais and Chevais & Thomas reported the production of mutations in *Drosophila* by means of sulphonamides. The effect is on the borderline of statistical significance, and confirmation would be desirable. It is noteworthy that the only series in which the increase in mutation rate was striking (eight sex-linked lethals in 466 chromosomes) is a sample of sperm from late broods of males which had been daily given fresh virgin females. This suggests preferential action on immature germ cells, a possibility which would be in agreement with the known role of sulphonamides as competitive analogues of an essential metabolite.

In general, the test of metabolite analogues, especially of analogues of the purines and pyrimidines contained in nucleic acid and of amino-acids, seems a promising line of investigation. So far, it has hardly been taken up except in the just discussed experiments of Thomas & Chevais. Pontecorvo (unpubl.) tried benzimidazole, an analogue of adenine, on *Aspergillus* and *Drosophila*. In *Aspergillus* many aberrations were obtained, but their genetical nature could not be established; in *Drosophila* the result was negative. Fries & Kihlman (1948) obtained biochemical mutations by treating conidial cultures of *Ophiostoma multiannulatum* with the purine compounds

theophylline and caffeine, at least one of which—caffeine—interacts in the fungus cell with adenine. It is, however, not clear whether these two compounds had acted as true mutagens or by selection. Caffeine was also effective in mutation tests on bacteria (Demerec, 1949). Acriflavine, which forms stable salts with nucleates, produced mutations in *Drosophila* (Demerec, 1949; see Table 4). It also induced hereditary changes in yeast (Ephrussi, 1949) and bacteria (Witkin, 1947); but their genetical basis is not clear, and in yeast at least appears to be cytoplasmic rather than nuclear.

The assumption that metabolism influences mutation rate seems plausible *a priori*, and is in keeping with observations on the fluctuations of spontaneous mutability. Stubbe & Döring (1938; Stubbe, 1940) reported that a deficiency of nitrogen, sulphur or phosphorus increased mutation frequency in *Antirrhinum*. The essential factor was shown to be the disturbed balance of minerals, and not simply the deficiency of one particular mineral. Milani (1946*a*, *b*) altered the mineral balance in *Drosophila* food by adding an excess of phosphate ions. The result was encouraging: 477 chromosomes from the treated series contained 13 lethals (=2·7%), as compared with 3 in 545 control lethals. Moreover, three males yielded several lethals (personal communication). Buzzati-Traverso (1947) repeated the experiment, but was unable to confirm Milani's results. If, indeed, this result had been due not so much to an excess of phosphate ions *per se*, but to a change in the balance of food minerals, even slight differences in the food mixtures used by these two authors might account for the discrepancy between their results. In view of the theoretical and practical importance of the relationship between mutation rate and metabolism it seems important that experiments of this nature should be repeated, using if possible synthetic and sterile food mixtures. An attempt by the reviewer (unpubl.) to influence mutation rate by way of metabolism through adding dinitrophenol to the food of *Drosophila* larvae gave a negative result.

Zamenhof (1944, 1945) reports that the germinal mutation rate of the unstable gene *mt-3a* in *D. virilis* was significantly *decreased* by any one of the following treatments: raising the larvae on food containing 0·055–0·1% $CuSO_4$; raising them on food containing 0·5 M-NaOH solution; treating eggs, young and old larvae with ammonia. Since the author does not believe that the chemicals actually reached the gene molecule directly, he attributes the effect to general physiological disturbances, and he believes that many physical and chemical disturbances would act in a like manner. The discrepancy between his results and those of earlier workers who had claimed increases in mutation rate through copper sulphate (Magrzhikovskaja, 1938; Law, 1938) and ammonia (Lobashov & Smirnov, 1934; Lobashov, 1937) is interpreted as due to selection which keeps stable genes near the minimum, and unstable ones near the maximum of their possible range of mutability. In this connexion it is interesting to recall that a similar difference between the mutation rates of stable and unstable genes was found when their dependence on temperature was studied. While the mutation rate of stable genes rises with increasing temperature (Muller, 1928; Timoféeff-Ressovsky, Zimmer & Delbrück, 1935), that of unstable genes

remains unchanged (Demerec, 1932) or decreases (Fabergé & Beale, 1942). These observations make it appear doubtful whether mutations of stable and of unstable genes are similar in nature.

Treatment of *Drosophila* eggs and larvae with proteolytic enzymes (Zamenhof, 1943) resulted in high mortality, but not in an increase in the frequency of sex-linked lethals among the survivors.

Heavy water (deuterium oxide), introduced into *Drosophila* larvae by feeding or injection, had no mutagenic action (Zamenhof & Demerec, 1943).

Emerson (1944) made the highly interesting attempt to induce mutations in *Neurospora* by means of serum from rabbits which had been immunized against the fungus. A number of variants were obtained, and those which were tested were found to differ from the normal parents by a single gene. It is not sure, however, whether the effect was due to the antibodies against *Neurospora* or to some other constituent of the serum. As far as I am aware, the experiment has not been repeated. This is to be regretted, because the demonstration that mutation can be produced by antibodies would shed new light on the nature of the gene and its reduplication.

In bacteria, specific hereditary transformations by chemical means have been successfully carried out by Avery, MacLeod & McCarthy on *Pneumococcus* (1944; McCarthy, 1946), and by Boivin and his collaborators (Boivin, Delaunay, Vendrely & Lehoult, 1945; Boivin, 1947) on the *coli* bacillus. In both cases the transforming principle was found to be the polymerized desoxyribonucleic acid derived from the strain whose characteristics were subsequently induced in the treated one. At the present state of our knowledge of bacterial genetics and of the nature of the self-duplicating units in the cell it cannot be decided whether this transformation can properly be classed as the induction of a specific gene mutation by a chemical substance.

Non-specific hereditary changes in bacteria have been produced by a great number of substances. Most of these results have already been mentioned. With the exception of Tatum's work (1947), in which biochemical mutants were scored, mutation frequency in these experiments (Demerec *et al.* 1947; Witkin, 1947; Latarjet, 1948; Bryson, 1948, 1949; Demerec, 1949) was determined by scoring the number of phage-resistant individuals derived by treatment from a population of sensitive *Escherichia coli*, strain B. Among the effective substances were some which have given positive results also with *Drosophila* (nitrogen mustard, sodium desoxycholate, acriflavin); others which have not yet been tested on *Drosophila*, e.g. several carbamates and even so simple a substance as lithium chloride; finally, one, colchicine, which has given negative results in experiments with *Drosophila* (Law, 1938; Hadorn & Niggli, 1946). The exact genetical nature of these induced permanent changes in bacteria is difficult to assess. I fully agree with the following statement by Witkin: 'In addition to confirmation provided by tests on other organisms, the use of other mutations in *Escherichia coli*, entirely independent of the phage-resistant system, would constitute a valuable check on results obtained with specific mutagens.'

Incontrovertible evidence for the genic nature of chemically induced bacterial mutations would be gained if such mutations could be induced in Lederberg's strain of *Escherichia*, in which they could be subjected to genetical tests (see § III).

7. *Combination treatment*

A method which has come into use in recent years is combination treatment with two different mutagenic agencies applied in succession. Experiments in which both agencies were radiations fall outside the scope of this review. Tests in which one or both agencies were chemicals are still rare. Oehlkers (1943), as has been reported before, combined different inorganic and organic substances for the production of translocations, and found marked synergistic effects. Dotterweich *et al.* (1939, 1940) and Dotterweich & Schmidtke (1941) claimed that *Drosophila* which for several generations have been fed on follicular hormone or certain other substances react to X-radiation by increased mutation rate; Kanellis, who repeated the experiment with follicular hormone, could not confirm this (1943). Addition of ferrum oxydatum saccharatum to the food of *Drosophila* (Buchmann & Hoth, 1937) increased the mutagenic effects of X-rays to an extent which according to Buchmann & Zimmer (1940) is significantly higher than would be expected from the increase in absorption of the radiation. E. Paterson (personal communication) treated tissue cultures and fern spores with a combination treatment of urethane and X-rays and found that urethane, when applied first, suppresses the inhibitory action of X-rays on mitosis. D'Amato & Gustafsson (1948) combined X-radiation of barley seeds with various types of chemical pre-treatment and found both quantitative and qualitative effects on mutation rate. Karnofsky, Burchenal, Ormsbee, Cornman & Rhoades (1947) report that X-radiation of mice interferes with the lethal effects of subsequent injections of nitrogen mustard. On the other hand, Auerbach (1946) obtained a strictly additive mutation rate when *Drosophila* males were given mustard-gas treatment on one day and X-radiation on the following. Swanson & Goodgal (1948) treated conidia of *Aspergillus* with low doses of nitrogen mustards, which by themselves were unable to increase mutation rate, and found that the treatment markedly increased the mutagenic effects of subsequent radiation with ultraviolet light. According to Kaufmann & Gay (1948) combination treatment with infra-red radiation and nitrogen mustard appears to increase the frequency of mustard-induced rearrangements in *Drosophila*. So far, these data are too isolated and scarce to allow of interpretation; but systematic investigation with the combination method may well prove very fruitful in the analysis of the mechanism of chemical as well as of physical mutagenesis.

V. CONCLUSIONS

The work on chemical mutagens is still in its infancy. Two major problems are raised by it. (1) What is the requisite constitution for a chemical mutagen? (2) What is the mechanism by which a chemical mutagen produces its effect? It is very possible that neither question will allow of a single answer. Different groups of chemicals,

each characterized by different common features, may act in different ways. Auerbach *et al.* (1947) (see also Auerbach, 1949) have given reasons for supposing that the mustards and other mutagenic vesicants and lachrymators act by a more or less direct transfer of energy to the chromosome. If Pullmann & Pullmann's (1946) hypothesis on the action of chemical carcinogens should be proved correct, energy transfer might also form the basis of carcinogen-induced mutation. Niggli (1946) and Hadorn *et al.* (1949) express the same idea for phenol by classifying it as a 'Treffergift' ('hit poison', see Jordan, 1939). Without further experimentation, especially on the relationship between dose and effect, these assumptions, however, cannot be considered as more than useful working hypotheses.

Other chemical substances may act in a more indirect manner, by influencing metabolism, enzyme activity, permeability, viscosity, etc. With such substances a simple dose-effect relationship will not usually be expected; thresholds, quantitative and qualitative relationship with other constituents of the nucleus, selective action at particular stages in the life cycle of nucleus, cell and organism will often play a more decisive part. One may recall Stubbe's (1940) observations on the influence of mineral balance on mutation frequency in *Antirrhinum*, or the remarkable synergistic action of potassium chloride and urethane in producing translocations (Oehklers, 1943). Moreover, the same indirect mutagenic effect may be exercised by chemicals of very different constitution, a point which has been stressed by Oehlkers.

The analysis of the mutagenic action of mustard gas (§ IV, 2) has revealed a striking similarity to X-ray action. Two main differences have so far been detected: (*a*) a shortage, relative to a mutagenically equivalent dose of X-rays, of large rearrangements, and (*b*) the ability to induce deferred mutation. Auerbach, *et al.* (1947) and Auerbach (1947*b*, 1949) have suggested that the latter peculiarity may be a consequence of the relatively small amount of energy made available to the chromosome by a chemical 'hit' as compared with an ionization. Insufficiency of available energy may conceivably account also for the shortage of rearrangements, but other explanations are equally plausible, and a decision has to await further experimentation. The fact that translocations fall more markedly short of expectation than deletions suggests that influences on the reunion of broken ends play a part.

There is no necessity to assume that all or even many chemical mutagens share the peculiarities of mustard action. Hardly any data are yet available on this question. Demerec (1948) reports that gonadic mosaicism was found after treatment of males with dibenzanthracene; without further details the significance of this finding cannot be estimated, but frequent occurrence of gonadic mosaics might suggest that this substance, too, may act in a deferred way. It has already been pointed out that Demerec's data on dibenzanthracene and other carcinogens suggest that some of these substances may be more effective than mustard gas in the production of chromosome rearrangements. Strong (1949), on the other hand, claims that none of the mutations which occurred in mice after treatment with methylcholanthrene was connected with

chromosome abnormalities; but it is difficult to see how this can be established without cytological examination.

The comparative analysis of the action of chemical mutagens opens up a wide field for research. Its theoretical importance for the understanding of gene and mutation is evident; but there is also a practical side to it. Work in this field may result in the detection of suitable substances for applied mutation work. Already, now, Gustafsson & McKey (1948) have shown that nitrogen mustard may be used with advantage instead of X-radiation for the production of mutations in agricultural plants. Even more noteworthy is their claim that certain mutants are produced preferentially by mustard treatment as compared with X-rays. Although this observation does not necessarily imply a specific action of nitrogen mustard on selected loci (see § IV 2 (*f*)), it has significant practical implications; specificity of mutagenic effect, whatever its genetical basis, may allow artificial mutation to be directed, at least to a certain extent.

The greatest achievement, both from the theoretical and the practical point of view, would be the chemical production of specific mutations in the proper sense of the word, i.e. the planned attack on specific loci, causing them to mutate in a desired direction. The bacterial transformations, discussed above (§ IV, 6), appear to have attained this in effect, although not necessarily by mutation proper. For higher organisms we are still as far as ever removed from this goal. Theoretically, as suggested by Hindle & Pontecorvo (unpubl.), the use of antibodies against individual loci would seem a promising method; in practice it offers great difficulties.

The possibility that naturally occurring chemical mutagens may have played a part in evolution has been discussed by Auerbach *et al.* (1947) (see also Auerbach, 1949) and by Demerec (1949). Whereas the former authors stress the constructive role which may have fallen to these substances, Demerec emphasizes their potential destructive influence on the stability of the species and enumerates factors apt to counterbalance these negative effects. If, as seems probable from the data presented in this review, the genes respond by mutation to chemical influences even of simple and naturally occurring substances (allyl *iso*thiocyanate occurs in *Brassica* plants, phenols are produced in the animal organism), we must expect that these substances and the sensitivity of the genotype to their action have been ends as well as means of evolution, and that there has been selection of mutagens and of gene sensitivity resulting in the most favourable balance. This would provide evolution with a very flexible and nicely adjustable mechanism for regulating its own speed and possibly to some extent even its own direction. Research in the new field of natural chemical mutagenesis is of the greatest interest; but it will be laborious and difficult, because it is to be expected that the balance between natural chemical mutagens and the reactivity of the genotype to them will differ from species to species, possibly even from race to race, or from ecotype to ecotype.

VI. SUMMARY

The general methodological requirements for work with chemical mutagens are the same as for general mutation work, with special emphasis on questions of concentration, penetration, possible indirect or delayed effect, differences in susceptibility between individuals, strains and species. In work with weak mutagens the fluctuations of spontaneous mutability should be reduced to a minimum and should be included in the estimate of error.

The manner of application of a chemical substance depends on the type of substance tested and the type of organism and tissue to be treated. Various methods are discussed. *Drosophila* is still the most suitable object for a genetical analysis of mutagenic effects; for complete cytological analysis plants are preferable. Work on mice or other warm-blooded animals has theoretical as well as practical importance, but offers difficulties to quantitative treatment. Micro-organisms have great advantages for the detection of mutagens, especially those species in which genetical methods for the testing of suspected mutations can be applied. Results gained with one organism cannot be transposed to another without test, and quantitative comparison between data gained on different organisms or even different cell types of the same organism is not admissible. A number of experiments in which chemical and physical mutagenic agencies were applied in combination have given interesting results which suggest that this method may prove a useful tool in the analysis of mutagenesis.

A group of highly toxic vesicants, of which mustard gas is the best known representative, are powerful mutagens. In *Drosophila*, up to about 24% sex-linked lethals have been produced with mustard gas, as well as small and large rearrangements. The mustards have proved efficient mutagens also in barley, fungi and bacteria. Two other potent vesicants, lewisite and chloropicrin, gave negative results in mutation tests. So did osmic acid and picric acid, which resemble mustard gas in being fixatives. A lachrymator, mustard oil or allyl *iso*thiocyanate, is a definite, though weak mutagen. Two other lachrymators, chloracetone and dichloracetone, probably are slightly mutagenic.

Analysis of the effects of mustard gas and nitrogen mustard has so far produced the following results: in *Drosophila*, lethals are scattered along the *X*-chromosome in a similar way as X-ray lethals. Lethals on the second chromosome are 4–5 times as frequent as sex-linked ones. The frequency of recessive lethals is highest in sperm which becomes available for fertilization about 6 days after treatment. Production of mutations in females is difficult. Doses which increased mutation rate in treated ova did not induce mutations in untreated sperm which, through fertilization, had been introduced into these ova. About 20% of the sex-linked lethals from treatment of males are caused by small deficiencies. Large rearrangements are less frequent after mustard treatment than after a dose of X-rays which produces the same frequency of sex-linked lethals. The shortage of translocations is more pronounced than that of large deletions. One very abnormal type of segregation in the progeny

of a treated male points to a centromere effect of the treatment. Treatment of heterozygous embryos results in a great increase in the frequency of somatic crossing-over. Visible mutations do not seem to differ from those produced by X-radiation in either relative frequency or type. About 50% of the visible mutations in the progeny of treated males are mosaics. Apparent semi-lethals were often found to be caused by gonadic mosaicism for a lethal. Visible mosaics occur also in the progeny of treated females. In several cases the same mutation occurred as mosaic in two successive generations derived from a treated male. These observations are most plausibly interpreted by the assumption that mustard gas can produce delayed localized effects. In *Tradescantia* the cytological effects of mustard gas seem indistinguishable from those produced by X-rays of low intensity; but data on *Vicia* suggest that in plant material, as in *Drosophila*, the two mutagenic agencies may differ in effect. In barley, nitrogen mustard treatment favours the appearance of certain hereditary types; it is not known whether this is due to specific action on certain loci.

Phenol was found to increase the rate of visible mutations in *Antirrhinum*. Applied to excised ovaries of *Drosophila* it produced high frequencies of autosomal lethals. It is not yet decided whether phenol itself or some contaminant was responsible for the effect. A striking feature of these experiments is that some lethals occurred repeatedly in different ovaries and different series, suggesting a specific action of the treatment. Many phenols, phenol derivatives and related compounds can produce chromosome breaks in *Allium* roots.

Urethane, especially in combination with potassium chloride, induces translocations in *Oenothera* and other plants, and mutations in *Drosophila*.

In the progeny of mice which for a number of generations had been treated with methylcholanthrene, variants appeared, many of which were shown to be hereditary. Some were identical with known laboratory mutants, others were reversals to wild type, still others were new aberrations. Several hereditary variants were found in a small population of mice after treatment with dibenzanthracene. The frequency of sex-linked lethals was increased in the progeny of *Drosophila* males which had been treated with aerosols of various carcinogens. The number of lethals which were connected with rearrangements was strikingly high.

Formalin increases mutation rate when mixed with the food of *Drosophila*, but does not appear to do so when applied as vapour. In bacteria, treatment of the medium with hydrogen peroxide increases the toll of permanent variants in bacteria, although treatment of the bacteria themselves has no such effect.

Sulphonamides have been reported to increase mutation rate in *Drosophila*, the effect being most marked in sperm which was not mature at the time of treatment. Disturbances of the mineral balance in the nutrient medium increases mutation frequency in *Antirrhinum*; there are indications that the same may apply to *Drosophila*. Dinitrophenol mixed with the food of *Drosophila* did not influence mutation rate. Heavy water and proteolytic enzymes failed to produce mutations in *Drosophila*.

Copper sulphate and low pH *decreased* mutability of an unstable gene in *D. virilis*.

Mutation rate in *Neurospora* was increased after treatment with serum from rabbits which had been immunized against the fungus; it is not clear whether the antibodies against *Neurospora* were the effective mutagen.

Specific transformations of bacteria have been produced by means of desoxyribonucleic acid from one strain applied to a second; the genetical basis of these transformations is not known.

Unspecific permanent variants in bacteria have been produced by a number of substances, carcinogens as well as non-carcinogens.

Theories concerning the mechanism of chemical mutagenesis cannot be more than working hypotheses at the present state of knowledge. It is possible that the mustards and some other compounds may act like X-rays by transferring energy to loci on the chromosomes. Some of the peculiarities of mustard gas action could be explained if it were assumed that the amount of transferred energy sometimes is sufficient only for the production of an unstable premutation. Other chemical mutagens may act indirectly by influencing metabolism or by disturbing the physicochemical conditions of the cell. It seems likely that chemical mutagens have played a part in evolution, and that their production and the sensitivity of the genotype to their action have been subject to selection.

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