New Features of Protoplasmic Structure Observed in Recent Electron Microscope Studies

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With six plates (figs. 1–6)

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SUMMARY

1. This article discusses current electron microscope specimen techniques (particularly thin sectioning) and various special aspects of protoplasmic structure. It is not a general review and deals only with subjects studied in this laboratory. Dimensions specified are approximate only.

2. As regards sectioning (which is inevitable for the study of fine cell structure in most complex tissues, by whatever microscope) electron microscope practice has two considerable advantages over light microscope practice: (a) osmium tetroxide solutions alone (without addition of dichromate, &c.) are excellent for cytoplasmic fixation; (b) after cutting, sections receive no further treatment (such as de-waxing and re- and de-hydration). Moreover, criticisms regarding vacuum drying of electron microscope specimens are irrelevant to electron microscope studies of sections. For high resolution, sections must be cut at 0.05 μ or less and examined without removal of embedding medium. Such sections are obtained by embedding in medium-hard plastics and cutting on a glass knife. Specially sharpened blades can be used instead of glass, but it is doubtful whether waxes can be substituted for plastics.

3. Many, possibly all, animal and plant flagella contain two similar central sub-fibrils surrounded by a ring of nine fibrils different in size and chemical composition from the central pair. As far as is known at present, mammalian sperm are unique (a) in containing a second concentric ring of nine sub-fibrils and (b) in possessing a double-helix sheath round the axial sub-fibrils of the tail. Bacterial flagella consist of single fibrils each equivalent to one of the component sub-fibrils of a multi-fibrillar flagellum. They often occur in bunches, but so far no intermediates have been found between these bunches and the sheathed '9+2' flagella of animals and plants. Vertebrate striated muscle consists of sub-fibrils which are (very roughly) 100 Å thick,

250 Å centre to centre and which in resting amphibian muscle have a 400 Å periodicity; in cross sections these sub-fibrils are packed solid (not in hollow cylinders) and in a fairly regular array.

4. Nuclear, cell, and mitochondrial membranes appear double in cross sections (150–300 Å thick); this may be due to the dissolving away of internal lipoid leaving two outer protein sheets, but none of these membranes is thin enough to contain simply a bimolecular lipoid layer. Electron microscope studies of striated cell borders confirm that in some sites there may be distinct filaments of variable length and in others closed ducts, or rods, covered distally by a smooth membrane. One border described contains distinct filaments which join basal mitochondria. It is not yet certain whether the complex internal 'double-membranes' of sectioned mitochondria arise from tubes, or paired sheets, or both.

5. When sectioned after freezing-drying or buffered osmic or formal fixation, the cytoplasm of many protein secreting cells in vertebrates is full of double membranes, like those of mitochondria, and of equally uncertain origin. Sections of many other cells show similar structures, varying in thickness from 75 Å–600 Å. There is some evidence that they are associated with cytoplasmic ribonucleic acid.

**INTRODUCTION**

It may well be objected that the above title does not embody the proper approach to cytology, which should be by subjects rather than by techniques. It is, however, simply a convenient way of stating that this article will describe several new features of protoplasmic structure at levels of organization below the limit of resolution of the light microscope, and of a kind which either have not been, or cannot conveniently be, studied by any other technique than electron microscopy. And since techniques are so important, they are briefly reviewed in the first section, after which various specialized topics of protoplasmic structure are discussed. Any attempt at completeness either in text or references is impossible within the scope of this article and hence only those subjects and methods are considered which have been investigated in this laboratory during the last few years.

**METHODS**

Those whose researches have required the routine cutting of 2–3 μ sections by conventional procedures will appreciate the difficulties involved in preparing sections between fifty and one hundred times thinner, as is necessary for high resolution electron microscopy. Fortunately the technical problems have not proved too severe and at the time of writing it is relatively easy to obtain sections about 350 Å thick as shown in figs. 3, 4, 5, and 6, a. These were prepared as follows: (a) fix in 1 per cent. osmium tetroxide solution buffered to pH 7.2 with acetate-veronal (Palade, 1951); (b) embed in butyl methacrylate (Newman, Borysko, and Swerdlow, 1949); (c) section at a microtome setting of 350 Å, using a glass knife (Latta and Hartmann, 1950) and a modified Cambridge rocking microtome identical in principle with the early models manufactured in the late nineteenth century. Techniques of this general kind are now in use in numerous laboratories and though they will doubtless be further improved, they nevertheless represent a substantial
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advance over what was possible a few years ago. The great problem is fixation, for minor alterations of the protoplasm, which would be invisible in a light microscope, appear as gross distortions at magnifications of 50,000 to 100,000. There is still only the dimmest knowledge of how and why osmium tetroxide is a good fixative; its special properties have nowhere been more lucidly described than by Baker (1945). They have recently been reinvestigated by Porter and Kallmann (1953). We have found that buffered formaldehyde solutions also make quite good fixatives for the electron microscope level of investigation (fig. 6, B, C).

Everything which has so far been published proves that it is impossible to dissolve away the embedding material without destroying the finest details of protoplasmic structure which the electron microscope is capable of revealing (Hillier, 1949). We have made careful comparisons (in ester wax sections) of mouse intestinal brush borders with wax remaining and wax removed which leave no doubt about this point. If the embedding material can be gently volatilized in the electron beam (without fouling the microscope!), well and good (Watson, 1953). Otherwise the presence of embedding medium and the slight consequent loss of contrast must be tolerated, but with adequately thin sections and with tissues whose natural opacity has been enhanced by osmic fixation this loss is small. Dissolving away the embedding medium is perhaps permissible where it is desired only to reveal rather distinct and characteristic inclusion bodies such as plant and animal viruses; and if the embedding medium is to be removed, then still further contrast can be achieved by metal shadowing, as the pictures of Wyckoff and his co-workers show. But the fine structure of the protoplasm in cells with embedding medium dissolved away is so adversely affected that the results are sometimes no better than could be achieved with the light microscope.

Regarding the examination of whole organisms or suspensions of granules or fibres little need be said, as relevant techniques have been discussed by Cosslett (1951). The chief difficulties are: (a) the centrifugation and (b) the contact with distilled water which may be involved in making clean preparations, and (c) the distorting effects of drying (e.g. flattening). All these troubles can be avoided if desired. Clean preparations without contamination can readily be obtained by a draining procedure such as that used by Valentine and Bradfield (1953). Distilled water has little or no harmful effect after proper fixation, which both renders proteins insoluble and destroys membrane semi-permeability. Drying effects can be overcome by freezing-drying, which we have used satisfactorily for several years, or by Anderson’s (1950) critical point method. Williams (1953) and Backus (1950) have developed elegant techniques of freezing-drying and spray-drying. But, in fact, the flattening caused by drying is a sine qua non for the successful examination of many dense structures (e.g. sperm tails), which without it would be quite opaque at the voltages in current use.

Much criticism hitherto expressed or implied regarding the techniques of biological electron microscopy is without foundation as far as the greater part
of the field is concerned. Only in the examination of suspensions of organisms or particles is the drying inherent in electron microscopy a real difficulty and even here there are satisfactory means of overcoming it when desired, as discussed above. For the examination of the vast majority of tissues, by whatever microscope, fixing and sectioning is inevitable, for the size, complexity and dependence on blood-supply of the tissues concerned strictly limit the information which can be obtained from the examination of their living cells (considerable though this sometimes is). For sections, the vacuum desiccation inherent in current electron microscopy constitutes no drawback whatever. Indeed it constitutes an integral part of one of the best methods of preparing tissues for sectioning—the Altmann-Gersh freezing-drying technique. The basic problem becomes the old and ever-present one of fixation, and here the electron microscope procedures, far from being at a disadvantage compared with light microscopy, have much in their favour. The best cytoplasmic chemical fixative known at present is a buffered solution of osmium tetroxide, but this is very little used in light microscopy because it often leaves tissues in a crumbly condition difficult to section, and in an altered chemical condition difficult to stain. Neither of these tendencies is detrimental in current electron microscope procedures (as far as cytology, as distinct from cyto-chemistry, is concerned), and indeed the osmium is an excellent electron stain. Moreover the electron microscope procedures involve only fixation, embedding, and sectioning; apart from examination in the microscope itself, at normal low beam intensities, there is no further treatment after sectioning. They thus avoid all the stages of de-waxing, hydration, staining, and dehydration in which artifacts may be introduced during the course of normal cytological practice for the light microscope.

The following is a summary of the techniques used in making the preparations illustrated in the plates.

**Fixation.** Figs. 1, A, E, and 2, A–C: 2 per cent. formaldehyde solution. Figs. 1, B–D; 2, D, E; 3; 4; and 5: 1 per cent. osmium tetroxide solution buffered to pH 7.2–7.4 with acetate-veronal (Palade, 1951). Fig. 6, B, C: 2 per cent formaldehyde solution similarly buffered.

**Sectioning.** Figs. 1, A–D; 2, D, E; 3; 4; 5; and 6 A: butyl methacrylate at a microtome setting of 0.035μ. Fig. 6, B, C: ester wax at 0.14μ. (All with embedding medium undissolved.)

**Shadowing.** Figs. 1, E and 2, A–C: 60 per cent. gold and 40 per cent. palladium alloy at approximately 30° to the horizontal.

**Microscopy.** Siemens electron microscope at 70 kV.

**Fibrillar Contractile Structures—Flagella 1–5 and Muscle**

**Flagella.** It has long been known that animal and plant flagella contain fibrillar protein oriented parallel to their long axes, but the detailed arrangement of the fibrils is only just being elucidated. In the case of bacteria, the resolution limit of the light microscope is such that there has even been contro-
versy (reviewed by Knaysi, 1951) over the existence of bacterial flagella. The contributions of electron microscopy in this field may be summarized thus:

1. Number of fibrils. All animal and plant flagella which have so far been examined in detail contain a definite and relatively small number of component fibrils which are between 100 Å and 200 Å thick. In many cases (cock sperm, Grigg and Hodge (1949); Ulothrix, Fucus and Chaetomorpha sperms, Paramecium, an unidentified mollusc, frog, human ciliated epithelium—references in Manton (1953); cilia of Mytilus edulis (personal observation); sperms of several species of slug and one species of cockroach (Cameron) and of Paracentrotus lividus (fig. 1, A), it is clear that the total number of component fibrils is 11—an outer ring of 9 similar fibrils and 2 other fibrils, probably thinner and centrally placed and certainly different chemically from the other 9. In particular the 2 fibrils are more sensitive to distilled water and digestive enzymes, so that it is all too common to find only 9 fibrils remaining in a macerated flagellum where there should in fact be 11. It can be claimed that any number just below 11 should be regarded as false owing to loss of fibres, but the admittedly widespread distribution of the 9+2 pattern must not be allowed to blind us to the possibility of other patterns. Indeed, it has recently become clear that a more complex pattern does exist in some, possibly all, mammalian sperm mid-pieces, where there are two concentric cylinders of nine fibrils plus the usual central pair—20 in all (fig. 1, B–D; Challice, 1953; Bradfield, 1953). The fibrils of the inner cylinder are more slender than those of the outer cylinder and of about the same thickness as the central pair. The exact doubling of the peripheral fibrils only serves to emphasize the significance of the number 9, which is at present a mystery. It is, however, noteworthy that in bull and ram sperm, for instance, the fibrils are gathered into 3 bundles in the neck region, each connecting with one of the 3 granules set in the base of the head. It is not known whether the bundles are similarly constituted, but at least the number of peripheral fibrils (9+9) is exactly divisible by the number of bundles (3)—as if there were 3 primary contractile units, each subdivided to facilitate finer gradation of effort. Three such units would presumably be the minimum apparatus necessary to produce 3-dimensional waves (whether these generate a circle or a very flattened ellipse when viewed end-on) in a sperm tail consisting of straight contractile fibrils. The chemical nature of the tail helix (see below) argues against its being contractile, though this point has not been finally settled yet.

Frey-Wyssling’s (1953) recent survey of sperm tail structure is unfortunately misleading. The statements regarding 8 fibrils in a tube around one central fibril (presumably for bull sperm, though this is not specifically stated), 6+6 for ram sperm, and 9 for sea-urchin sperm are all incorrect (see figs. 1, A–D for two of these sperm).

The most complicated flagella apart from mammalian sperm-tails are probably the so-called flimmer-flagella found in numerous Protista and in some cryptogam sperm. Despite studies by Houwink (Euglena), Manton (Fucus), and
in this laboratory (*Ochromonas*), the detailed relation of the fine lateral branches to the main axial fibres is not fully understood.

Although there may be chemical differences between them, the component fibrils of a flagellum usually have the same length, within a few m\(\mu\). Notable exceptions are found among fern sperm (fig. 1, e) where an elegantly tapered point results from the way in which the component fibrils end one by one until only a single fibril (or triplet?) runs to the extreme tip.

2. Sheaths and membranes. In all multi-fibrillar flagella which have been studied there are one or more sheathing membranes binding the fibrils together. This is particularly conspicuous in the fern sperm flagella mentioned above (fig. 1, e), where the sheath commonly becomes displaced in a peculiar and characteristic manner. Round mammalian sperm tails there is, in addition to a sheathing membrane, a stout double helix, composed at least partly of fibrillar protein, which we have found to be strongly resistant to o-2N hydrochloric acid and 6M urea, but dissolved by o-1N sodium hydroxide. This cortical helix occurs in all mammalian sperm studied up till now, including a marsupial sperm of which we have taken a few photographs (fig. 2, A), and has not been found outside Mammalia. It may therefore constitute one of their diagnostic characters.

3. Bacterial flagella. A bacterial flagellum consists of a single fibril of very even diameter, closely similar in size to one of the component fibrils of multi-fibrillar flagella, often originating from a basal granule and very distinct from a strand of mucus. No such flagella have been detected in viruses or *Rickettsiae*, so that this single fibril is presumably the basic evolutionary unit in the flagellar hierarchy. It displays just as wide an evolutionary radiation as do the more complex multi-fibrillar flagella. There may be a single polar flagellum, or one at each pole; or numerous flagella regularly arranged over the bacterial surface; or a bunch of flagella at one pole or at both poles (*Spirilla*). Or, finally, instead of hanging free, the fibrils (one or many) may be wound spirally round the elongated axis of the body (fig. 2, c) and attached at both ends: this is the interesting structural device which distinguishes the spirochaetes (Bradfield and Cater, 1952), there being only 1 fibril in the smallest (*Leptospiira*), from 4 to about 30 in genera of intermediate size (*Treponema* and *Borrelia*), and a bundle of more than a hundred in the larger species such as *Cristispira*.

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**Fig. 1 (plate).** A, Sea-urchin sperm. T. S. showing ring of 9 fibrils surrounding 2 thinner central fibrils. X 40,000. B, Bull sperm. Slightly oblique T. S. of mid-piece showing outer mitochondrial sheath, inside this a cylinder of 9 thicker fibrils (showing here as short rods on account of the oblique cut), inside them a cylinder of 9 thinner fibrils, and inside them a pair of fibrils very close to each other and similar in thickness to the fibrils of the inner cylinder (see d). X 45,000. C, Bull sperm. T. S. of tail distal to mid-piece, showing a ring of 9 fibrils around a central pair; there may be an intermediate ring of very fine fibrils, or the 9 conspicuous fibrils may be double, this is not certain yet. X 72,000. D, Bull sperm. Diagrammatic clarification of the inner 9 + 2 fibrils in B; suggested 3-dimensional aspect is given by widening the fibrils on one side. E, Bracken sperm, tip of flagellum. Component fibrils end one by one. The ends of the 9 main fibrils are indicated by arrows. Sheathing membrane artificially displaced. X 70,500.
FIG. 1

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But neither in the large spirochaetes nor in the *Spirilla* has any trace been found of a sheathing membrane surrounding the bundle or bunch of fibrils. In fact no intermediates whatever have yet been found between the collection of separate bacterial fibrils and the true multifibrillar flagellum with a well-defined membrane sheathing its 11 or more fibrils. It is hardly credible that this complex arose at a single step. Intermediates may of course have become extinct, but it will be interesting to hunt for them among the vast assemblage of present day Protista.

4. Mode of action. If we assume that the ring of fibrils in a multi-fibrillar flagellum are contractile, it becomes relatively easy to make hypotheses about the behaviour of the flagellum (or cilium) as a whole (Gray 1928), whether 2- or 3-dimensional. Plenty of physiological problems remain, and why there should be the odd combination of 9+2 is likely to be a puzzle for some time. But it is necessary only to postulate the power of linear shortening in the component fibrils of the ring in order to make simple hypotheses of movement.

On the other hand, it is by no means clear, in the case of a uni-flagellate bacterium, how waves could be propagated down a single fibril 100–200 Å in diameter, equivalent to one component fibril of the multi-fibrillar flagellum discussed above. It may be that the motif is repeated on a smaller scale and that each 100–200 Å fibril itself consists of a bundle of smaller fibrils in the manner of Dean Swift's fleas; but the process could certainly not go on *ad infinitum*, for the sub-fibrils within a 100–200 Å fibril would already be of the order of diameters of single protein molecules. The alternative (apart from passive waggling by a structure active at the base of the flagellum) requires the generation of waves by contraction within a large, single 'molecule' or string of molecules—either by local contractions passing down opposite sides out of phase (2-dimensional waves), or by local contractions passing round a helical structure such as is now favoured for some protein molecules (3-dimensional waves). The recent suggestion by de Robertis and Franchi (1951) that some bacterial flagella consist of a helical core inside a distinct sheath would provide a possible structural basis for the latter idea. We have not observed a similar structure in the bacterial flagella studied here, but this may be due to faulty technique or unsuitable species.

5. Non-fibrillar flagellum-like structures. In conclusion it is of interest to refer briefly to the remarkable filamentous appendages of Crustacean sperm, which look as if they might be constructed on the flagellum-pattern but are

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**FIG. 2 (plate).** A, Opossum sperm. Helix round tail, which is the paler structure underlying the head and mid-piece of another sperm, the latter being too dense to show structure. (These sperm were in poor condition when obtained.) × 17,500. B, *Notodromas* (Ostracoda) sperm. Empty tail sheath showing imprint of the 6 helical components originally inside (4 broad and 2 narrow). ×7,000. C, *Treponema duttoni* (African tick fever spirochaete, cultured in mice), after mild trypsin digestion which slightly disrupts (thereby displaying) the bundle of fibrils which is wound helically round the organism and can be seen passing alternately over and under the body in this shadowed preparation. When intact, the fibril-bundle is tightly wound round the surface of the organism. × 19,500. D and E, *Chironomus* salivary gland; detail of mitochondria (see fig. 3). × 205,000.
not. These sperm are said to be without true flagella and we have confirmed that in the three appendages of lobster sperm, for example, there is no sign of fibrillar structure of the kind or on the scale found in true flagella (Rothschild, 1952). Equally unusual are the tails (called heads by Retzius) of freshwater Ostracod sperm, where the customary fibre-bundle plan has been abandoned in favour of an extraordinary 6-thread spiral flex (fig. 2, b). Other Crustacean and some Arachnid sperms are equally aberrant in this respect, but recent electron microscope studies in this laboratory show that at least some insect sperms are very close to the 'archetype' tail structure (Cameron).

Muscle. Electron microscopical additions to our knowledge of the fine structure of striated muscle may be summarized thus:

1. Myofibrils can be resolved into fine filaments about 100 Å wide and showing a marked 400 Å axial periodicity.

2. Myofibrils are packed solid with these fine filaments disposed in regular array (Morgan and others, 1950), about 250 Å centre to centre, and are not hollow cylinders with walls one filament thick as was once thought possible. This was first demonstrated by Morgan and others (1950), and has been confirmed in this laboratory and by several other workers, particularly Huxley.

3. Draper and Hodge (1950) state that in toad muscle the axial periodicity in the filaments varies from 400 Å–250 Å, roughly in proportion to the sarcomere length and degree of contraction. They are tentative about this, however, and about differences in periodicity in different bands of a sarcomere, and there are obviously several important questions needing further investigation here.

Nuclear, Mitochondrial, and Cell Membranes, and 'Brush Borders'

The outstanding feature about this group of membranes is that each appears to consist not of a single layer of its constituent molecules, but of at least two distinct layers. To a certain extent this may simply be a direct confirmation (and extension to nuclear and mitochondrial membranes) of the double lipo-protein layer theory of cell membranes described by Danielli (1941), as Sjöstrand (1953 a, c) has suggested—the central lipoid being lost during the preparation of sections and the two outer protein layers remaining to produce the double structure observed. But the principle extends to cell walls which are not primarily lipo-protein in nature, as in the very interesting yeast cell wall investigated cytochemically by Northcote and Horne (1950). Here it appears that one layer consists mainly of a glucan and the other of a mannan plus lipo-protein (a varied mixture!). Probably the latter will be subdivided by future work.

Electron microscopy has so far shed little light on the internal structure of the nucleus, for a number of reasons which need not be elaborated here. The nuclear membranes of two amphibian oocytes have, however, been elegantly investigated by Callan and Tomlin (1951), who find each to consist of an outer, porous membrane (pores 400 Å diameter and 1,000 Å centre to centre) and an inner continuous membrane; both membranes contain relatively
insoluble protein and the outer in addition contains some lipoid. Double nuclear membranes have now been found (in sections) in such diverse sites as mammalian kidney and liver, insect silk glands and amoebae, but it remains to be seen whether these resemble chemically the oocyte nuclear membranes.

There has long been evidence for the existence of membranes round mitochondria. But even in 0.2 μ sections, mitochondria are too dense to reveal details of their structure. Only since it has become feasible to cut sections of the order of 0.05 μ, or less, has it been possible to observe the detailed structure of sectioned mitochondria. Sjöstrand’s micrographs show that the mitochondria of mouse kidney proximal convoluted tubules are bounded by a double membrane 160 Å thick, the two component laminae being 45 Å thick and 115 Å centre to centre. In addition these mitochondria contained ‘a system of internal double membranes oriented chiefly transversely to the long axis of the mitochondria’. Sjöstrand (1953c) states that at their outer edges the two constituent laminae of the inner double membranes are joined so that the space inside them is closed, but in other respects the inner and outer membranes are identical. In other words his mitochondrion resembles a double-walled money-box containing a stack of hollow coins (not necessarily circular).

This may well be the case in many mitochondria, but in various insect cells (salivary glands of Chironomus and Malpighian tubules of Galleria) which we have studied it appears likely that at least some of the internal double membranes of mitochondria arise from tubules and not from hollow disks or sheets. Mitochondria were not our primary object of study, but they are very conspicuous in these cells and in both sites the sectioned mitochondria showed (in addition to longer runs of double membrane) numerous small circles and ellipses (figs. 2, D, E; 3; 4). Clearly the latter may be tubes in cross section, but they may also be granules in cross section, and here only serial sectioning can decide. Both this and the important question of fixation artifacts will be discussed in the next section, but it is worth noting here that few structures could be better placed for fixation than the mitochondria in figs. 2, D, E and 3, which are only 2-3 μ from the outer cell border and hence from the fixative. Even slowly penetrating osmium tetroxide must reach them very soon after the cells come into contact with fixative, which in this case was immediately the animal was killed by decapitation.

Electron micrographs of the outer ends of retinal rods (Sjöstrand, 1949) and of chloroplasts (Leyon, 1953) show that both contain stacks of discoid lamellae. It is hard to believe that chloroplasts are, as stated by Leyon, devoid of outer sheathing membranes.

In contrast to the mitochondrial membrane whose existence, though postulated, was far from certain before it was identified in the electron microscope, the cell membrane, about which so much has been written, is often quite difficult to observe in electron microscope sections. Sjöstrand (1953c) states that in the proximal convoluted tubules of mouse kidney, the cell membranes
consist of two 60 \text{ Å} laminae (presumably protein) separated by 160 \text{ Å}. The latter is too great to be spanned by a bi-molecular layer of lipoid.

In some highly differentiated cells, the boundary membranes undergo elaboration at a level of organization where the electron microscope can reveal useful new or confirmatory information. Thus in the myelin sheath of axons, the micrographs of Fernández-Morán (1950) and Sjöstrand (1953b) have given direct confirmation (in general arrangement if not in detailed dimensions) of the multilayer structure predicted from X-ray diffraction. With regard to the interesting brush-borders which are common in absorptive epithelia, the rod versus pore controversy, dating from the mid-nineteenth century, rages as fiercely as ever (J. R. Baker, 1942; Granger and R. F. Baker, 1950). As Wigglesworth (1950) points out, it is very likely that both are true, in different cells. From comparisons of sections with and without embedding medium, we are certain that removal of the latter seriously disrupts such fine structures as these, and this factor may well account for the distinctly separate rods demonstrated by Granger and Baker (1950), Bretschneider (1952), and others. Sjöstrand (1953c) believes that the brush-border of mouse kidney proximal convoluted tubules consists of fine closely packed ducts, circular in cross section, filled with protoplasm, closed at each end, with ramifying tubes leading down into the cytoplasm from their inner ends, and covered by a continuous smooth membrane at their outer (distal) ends next to the lumen. On this view the spaces between the ducts do not communicate with the lumen of the kidney tubules and Sjöstrand does not therefore subscribe to the view that they are a means of increasing absorptive area.

It seems that a straight, closed 'duct', filled with protoplasm, might more simply be called a rod, but what matters is not nomenclature, but the thorough understanding of the structure, which Sjöstrand rightly describes as very intricate. We have studied the striking borders of insect Malpighian tubules, particularly in the waxmoth caterpillar (Galleria). It is very probable that, as Wigglesworth (1950) has shown for Rhodnius, there is a honeycomb border in the upper region of these Malpighian tubules and a true brush-border in the lower regions. Our sections are from the lower regions and have been examined without removal of methacrylate. There seems no doubt that, as Wigglesworth found for the corresponding zone in Rhodnius, the border consists of separate rods of variable length. It is unlikely therefore that they can be covered with a continuous outer sheet, and none has so far been seen. But more interesting than the outer border is the inner (proximal) end of each rod, which connects in many cases with a typical mitochondrion; a few clear examples of a junction between brush-border rod and underlying mitochondrion, as well as several 'near-misses', may be seen in fig. 4. Some of the mitochondria in question are full of the double membranes discussed above; others contain numerous

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**Fig. 3 (plate).** Chironomus salivary gland. L. S. outer 3–4 \( \mu \) of cytoplasm which is rich in tracheoles (empty circles) and mitochondria. The latter show conspicuous 'double membranes' (sometimes transverse, sometimes longitudinal, often branched), but also contain irregular circles or ellipses, which may be tubes or granules in T. S. (See Fig. 2, D, E.) \( \times 75,000 \).
FIG. 3

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FIG. 4
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irregular circles and ellipses which are probably oblique transverse sections through tubes (though they might of course be granules, and only serial sections can decide). Whether they prove to be derived from laminae or tubules, it is noteworthy that the double membranes extend from the basal mitochondria up into the brush-border rods, thus emphasizing the similarity of the latter to mitochondrial extensions or appendages. These basal mitochondria probably correspond to the 'basal granules' which have long been known and which are picked out by mitochondrial stains (J. R. Baker, 1942). We are of course using the characteristic internal structure which has just been shown to exist in mitochondria as a criterion for identifying new granules as mitochondria. In fact, this should be only one of several criteria, including vital staining and enzymological characteristics (reviewed in Bradfield, 1950) used to define these bodies, and with such diverse criteria available it seems time for a comparative study of whether all such features always exist side by side, or whether various combinations and gradations of properties occur among the great array of mitochondria-like cytoplasmic particles found in living cells.

Cytoplasmic 'Double Membranes'

Among the most interesting features of protoplasmic structure so far revealed by electron microscope study of thin sections are the so-called cytoplasmic double membranes (figs. 5; 6, b, c). They appeared at first as long fibrils (Hillier, 1949; Dalton and others, 1950), but were later found to be double and resolved into pairs of parallel lines (Bernhard and others, 1952; Sjöstrand, 1953a) and hence their current name. It is already evident that the total thickness of double membranes may vary considerably from tissue to tissue. For mouse pancreas the figure is approximately 600 Å, or 360 Å centre to centre (fig. 6, b, c); for mouse kidney proximal convoluted tubules 270 Å, or 190 Å centre to centre (Sjöstrand, 1953a)—though until it is proved that these structures are sheets and not tubes (see below) it is hardly admissible to take an unbiased average thickness; and for waxmoth caterpillar Malpighian tubules the total thickness may be as low as 75 Å, or 50 Å centre to centre (fig. 5). In the few tissues already studied there is, therefore, almost a tenfold variation in total thickness and it is questionable whether all double membranes, wide and narrow alike, have the same kind of organization. The thinnest which we have encountered—in caterpillar Malpighian tubules—are of the same order of thickness as the intramitochondrial double membranes in the same cells and thinner than the intramitochondrial double membranes of some other cells (mouse kidney and Chironomus salivary glands). In some protein-secreting cells such as those of mouse pancreas (Sjöstrand, 1953a, and

Fig. 4 (plate). Malpighian tubule of the caterpillar of the waxmoth (Galleria), L. S. showing small area at inner (proximal) end of the brush-border filaments. A few filaments are seen to arise directly from basal mitochondria and the 'double membranes' inside the latter extend up into their respective filaments. Some mitochondria contain circles and ellipses suggestive of tubes or granules in T. S. ×70,000.
fig. 6, B, c) and rat salivary gland there are such serried arrays of double membranes that the sectioned cytoplasm looks like a miniature railway marshalling-yard. In other cells, such as mammalian kidney, liver, and intestine and insect Malpighian tubules, the double membranes are present but less numerous. In mammalian neurones Hagenau and Bernhard (1953) find nothing quite like the double membranes of kidney or pancreas and that has been our experience with Chironomus salivary glands and caterpillar silk glands, which is rather surprising in view of the fact that mammalian protein-secreting cells possess abundant double membranes. However, our provisional interpretation of Chironomus salivary glands (fig. 6, A) is that the bulk of the cytoplasm consists of a tangled mass of ramifying tubules 500–600 Å in external diameter. Because of their convolutions none of the tubes runs very far in any one section and hence they do not give the same visual impression as ‘double membranes’; but if the latter turn out to be tubes rather than sheets (see below) the two structures may well prove to be of the same general kind.

As indicated above, these discoveries raise several problems. Do the double membranes seen in sections arise from paired sheets, or are they chance longitudinal sections of tubes? And does the parent structure in the embedded tissue exist as such in the living cells, or is it an artifact? (Even if it were, the observations cannot be dismissed as meaningless, for such intricate structure must reflect pre-existing complexity—either chemical or structural.)

With regard to the possibility of fixation artifact, it is noteworthy that very similar double membranes have been observed in exocrine pancreas cells fixed in buffered osmium tetroxide solutions, or by freezing-drying (Sjöstrand, 1953a), and in buffered formaldehyde solutions (fig. 6, B, c). The only differences are in minor details of spacing and thickness. The fact that two good (but very different) chemical fixatives, together with an excellent physical method of fixation, all give closely similar results provides strong evidence that the parent structure does exist in living cells. Structures of this kind naturally bring to mind the danger of myelin-figure formation. Palade and Claude (1949) have shown this danger to be serious when a wave of acidification advances through the tissue, favouring the formation from some phospholipoids of myelin figures, which are then fixed by the succeeding wave of more slowly penetrating osmium tetroxide. The acetate-veronal buffered fixative recommended by Palade (1951) and used in most recent electron microscope studies should minimize the formation of myelin figures, and during freezing-drying conditions are also unfavourable for their formation. To settle the reality of these structures by direct observation in living cells is unfortunately impossible in view of their size. But extensive arrays of them might well give rise: (a) to cytoplasmic striations visible in the light microscope; (b) in special cases to birefringence (Sjöstrand, 1953a); (c) to canals of the kind convincingly

**Fig. 5 (plate).** Malpighian tubule of the caterpillar of the waxmoth (Galleria). Similar to fig. 4, but the section is of a region near a nucleus (indicated by arrows in the bottom right-hand corner) where the mitochondria are smaller and the cytoplasm contains very narrow double membranes only 75 Å thick (50 Å centre to centre). × 95,000.
FIG. 5
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demonstrated recently by Ludford and Smiles (1953) in living polymorphic sarcoma cells, or of the kind reviewed by Bensley (1951) in his clear reassertion of the view that living cells contain complex arrays of canals and vacuoles similar in general, though not in detail, to the complex artifacts produced by classical Golgi methods. The parent structures of the double membranes observed in sections may well have been concerned in the production of classical Golgi pictures, but when the tube versus sheet debate is settled, it will undoubtedly be better to give them a name based on their form (in the manner of 'mitochondria') rather than to regard them as the new manifestation of the Golgi complex, for the latter term has outlived its usefulness.

With regard to the form of the structures which give rise to the double membranes seen in sections, no definite answer can yet be given. At first they were thought of only as fibrils (Hillier, 1949; Dalton and others, 1950), but later Dalton (1951) took the view that they were lamellae and Sjöstrand is of the same opinion. 'It is quite obvious that we are dealing with membranes and not filaments from the fact that they may be followed without interruptions through the whole basal cell zone, the chance to hit a filament so exactly along its entire length being negligible. In addition there have never been any indications of cross-cut filaments' (Sjöstrand, 1953c). The latter sentence is not true for our sections of mouse pancreas and Chironomus salivary glands (fig. 6, A), where there are plenty of indications of tubes cut in cross section; but these are only indications, for irregular circles and ellipses could be transverse sections either of tubes or of granules, and only serial sections can settle the matter. The validity of the first sentence is very hard to assess. Sjöstrand does not state the thickness of his excellent sections, but they appear to be of the same order of thickness as those shown in figs. 2, D, E, 3, 4, 5, 6, A, which were cut at a microtome setting of 350 Å. The probability of the parent structures being tubes, therefore, depends on the chance of getting a stretch several μ long of a 270 Å thick tubule in a 350 Å section through a cell containing an unknown number of such tubules—which may be oriented randomly or in a preferred direction. Again depending on the thickness of the section, paired sheets would not always generate the sharp double lines actually observed; the latter would appear only when the section cut the sheets at approximately a right angle (not more than 15°–20° either way). Obviously the relative probabilities are hard to calculate and it is better to defer judgement until the matter is determined by serial sectioning.

Porter (1953) has described in isolated tissue-culture cells the 'endoplasmic reticulum', which consists of irregular canals 500–1,000 Å wide, or in the case of vesicular units up to 6,000 Å wide. These structures may well belong to the same general family as those which give rise to the well-defined double mem-

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**Fig. 6 (plate).** A, Chironomus salivary gland. Similar to fig. 3, but the section is of a region near the cell centre which could be interpreted as a mass of tangled tubules of about the same thickness (600 Å) as the 'double membranes' in, for instance, mouse pancreas. X 33,500. B and c, Mouse pancreas (formalin fixed), showing cytoplasmic 'double membranes' at different magnifications. B, X 29,000. C, X 19,000.
branes. But we cannot agree that the latter are ‘easily identified’ with Porter’s endoplasmic reticulum in cultured cells (and have not been able to verify the reference to a paper by Palade and Porter (1952) where this claim is said to be substantiated). On the contrary, there are profound differences in degree, if not in kind, between the two types of structure. First, the endoplasmic reticulum is extremely variable in diameter in a single strand, and in a single cell type, with a lower limit of 500 Å (this could be partly due to flattening in the remarkably thin cells from which Porter obtains his excellent micrographs), whereas the double membranes are fairly regular in diameter in a single cell type with an upper limit, so far, of about 600 Å (mouse pancreas)—some being apparently as thin as 75 Å (fig. 5). Second, the endoplasmic reticulum is abundant in cultured cells of kinds (e.g. endothelial cells) where one would not expect to find abundant double membranes, for the latter are not a major cytoplasmic constituent in most cell types so far studied.

Only in sections of cells noted for their abundant protein secretion are double membranes known to be anything like as abundant as is the endoplasmic reticulum in almost all the cell types described by Porter.

If the double membranes seen in sections are homologous with anything in Porter’s cultured cells it is probably with the dense filaments of growing cells (his figs. 5 and 19), which because of their supposed relation to ‘growth granules’ are labelled ‘gg’. Porter makes the interesting observation that these filaments have a considerable intrinsic density, independent of osmic fixation, which is exactly our experience with pancreatic double membranes, which are almost as dense after fixation in formaldehyde as after osmic fixation.

Little can at present be said about the constitution and function of double membranes, or about their formation and growth—unless they do in fact correspond to Porter’s dense filaments (see above), which he believes to be formed by the aggregation of ‘growth granules’. Sjöstrand suggests that double membranes, like cell and intramitochondrial membranes, may be composed of an inner layer of lipoid, removed during preparation, and an outer coating of protein. This view is quite plausible, but there is no direct evidence for it yet. The thin double membranes seen for instance in fig. 5 might have the sort of bi-molecular lipoid layer postulated for cell membranes; thicker ones would need some more complex internal arrangement.

The other suggestion about the chemistry of double membranes is that they may contain, or be closely associated with, the cytoplasmic ribonucleic acid (RNA). Dalton suggested this in 1951 on the grounds that their distributions in exocrine pancreas and stomach chief cells roughly coincided with that of cytoplasmic basiphilia; and because basiphilia and double membranes were patchy in normal liver-cells, but more evenly distributed in hepatomas. Bernhard and others (1952), in producing further evidence for this view, have carried out some of the earliest experimental cytology at the electron microscope level. They found that double membranes were more abundant in actively secreting rat livers than in starved livers and in regenerating livers than in non-regenerating livers—exactly like the basiphil component of the
Cytoplasm. More recently they have found that in centrifuged livers the centrifugal displacement of the double membranes corresponds with that of the basiphilia. Porter (1953) suggests that his endoplasmic reticulum contains RNA, but as his cells were not strongly basiphil, in the way that peptic, exocrine pancreas and regenerating liver-cells are, it is hard to be sure about this. It is, however, noteworthy that strong basiphilia is apparently a feature of his dense particulates, which we believe are more probable counterparts of the double membranes seen in sections.

The evidence that RNA is located in double membranes is at present indirect—and there may always be another cytoplasmic component closely associated with them, confusing the results. Bernhard has pointed out that if the present view is confirmed, it will conflict somewhat with the biochemical bulk centrifugation studies, which indicate that cytoplasmic RNA is located in microsomes—small granules usually said to be 500–2,000 Å in diameter and not markedly anisodiametric. There would be three possibilities: (a) microsomes arise from double membranes by disintegration during centrifugation; (b) double membranes arise from microsomes during fixation; (c) both arise during preparation from some parent structure different from either. The evidence already presented that very similar double membrane pictures are obtained after three different fixatives is in favour of (a), but until the RNA content of double membranes is established we cannot be sure that (a) is the case.

**Conclusion**

Already in 1853, the first volume of the *Quarterly Journal of Microscopical Science* was concerned with several features of protoplasmic structure discussed here—muscle cytology (Lister) and the nature of flagella (Siebold). Mitochondria, however, had not yet been discovered, and cytologists were only just beginning the great edifice of theories and counter-theories about the structure of ‘ground-cytoplasm’, which was extensively demolished by Hardy and Fischer. Greatly superior fixation-methods are now available, but still the study of ground-cytoplasm is one of the most thorny corners of cytology. Discussion of it has been avoided here because it is doubtful whether anything useful can yet be said at the electron microscope level of investigation. We question the value of several recent studies in this field, especially of comparisons with relatively simple organic colloids which have very limited relevance to protoplasm. Also regrettable is the emergence of a forest of new terms for describing supposed components of ground cytoplasm—e.g. biosomes, chromidia, and leptons—often poorly defined despite their classical derivation. As Sjöstrand (1953c) points out, it is usually easier to preserve (and to check with the light microscope) the structure of the larger and more complex formed elements of protoplasm—mitochondria, cytoplasmic fibrils, and the like. After becoming thoroughly familiar with methods for the faithful preservation of these, we shall be in a better position to turn to the still finer details of the ground cytoplasm. And there is no shortage of formed structures
awaiting investigation—the ‘bottle-necks’ are all technical and two of the foremost are the difficulty of examining serial sections and of applying specific stains. However, in view of the fact that, as recently as 1939, the eminent microscopist, von Ardenne, gave good reasons for believing the whole technique of thin sectioning to be impossible by normal procedures, there are grounds for hoping that these new problems may also be solved in due course.

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