Whenever it is our intention to study objects that are visible with the light-microscope, it is reasonable to start with the living cell, to study the effects of fixation on its visible constituents, to analyse their composition by cytochemical methods, and then to explore their fine structure by the use of the electron microscope. A many-sided attack of this sort on a single kind of cell is much more likely to give valuable results than the independent study of different kinds of cells by different kinds of cytologists.

I shall begin by giving an account of a particular piece of work just completed in the Departments of Zoology and Human Anatomy at Oxford by Mr. J. T. Y. Chou, Dr. K. F. A. Ross, and Dr. G. Meek. Although in this account I shall deal only with a single cytoplasmic inclusion in a single kind of cell, yet you will notice the general implications of my remarks and their bearing on problems of fixation.

If we look at a living neurone from one of the ganglia of the common snail, Helix aspersa, by positive phase-contrast microscopy, we shall see a large number of spheres dispersed evenly in the cytoplasm. Each appears to have a dark rim. These spheres have a strong tendency to take up neutral red from very dilute solutions during the life of the cell. They are also easily dyed in life by methylene blue, brilliant cresyl blue, and Nile blue. Chou therefore calls them "blue" globules, to distinguish them from certain other globules in the cytoplasm (9). The interference microscope provides the information that the "blue" globules have a refractive index of about 1.41 (29).

A wide variety of cytochemical tests has been applied to the "blue" globules (10). No amino-acid, nucleic acid, or carbohydrate has been found in them. Sudan black, however, colours them strongly. The acid-haematein test is also positive: other tests for particular lipids are negative. It follows that the globules contain phospholipid, but 1.41 is too low a figure for the refractive index of any lipid. The most likely explanation of the facts is that the principal or only constituents of the globules are phospholipid and water.

It is known that in the presence of water, phospholipids have a strong tendency to arrange themselves in bimolecular membranes. It will be remembered that one end of the molecule is hydrophil, the other hydrophobic. Schmidt (31) supposes that the hydrophobic ends of two molecules associate with one another, while their hydrophilic ends associate with water. General intermolecular forces bring the bimolecules into lateral association with one another, so as to form a membrane. In his study of the bimolecular lipid membranes of myelin, Finean (16) concludes that the hydrophilic ends of the molecules are turned towards one another, but Schmidt's seems to be the more likely arrangement in the phospholipid membranes of cytoplasmic globules.

A globule composed of phospholipid and water must be supposed to consist of concentric bimolecular layers, separated by spaces filled with water. Since the "blue" globules have a more highly refractile rim, it is probable that the layers are situated towards the surface, while the centre of the globule consists mostly of water. If the layers extended throughout the globule, the bimolecules near the centre would need to be arranged (in sectional view) like the thick spokes of a small wheel. Intermolecular forces would be unable to hold widely divergent bimolecules in position. A considerable radius of curvature is necessary for stability. This may be the reason why phospholipid globules are often hollow.

Even when the radius of curvature is considerable, the bimolecules cannot have taken up positions dictated by intermolecular forces alone, for these would have brought each pair of molecules exactly parallel to the next, as happens when the long hydrocarbon chains of a paraffin become parallel in crystallization on cooling. Any force that brought the bimolecules into a more parallel position would tend to flatten the membrane and thus disrupt the sphere. Yet the globules are stable. The position is one of equilibrium, but there is a potentiality for change of form.

It was questioned whether any indication of
the hypothetical layered arrangement might be visible in electron-micrographs. To answer this, Chou and Meek fixed pieces of the ganglia of Helix in a sucrose-osmium mixture, buffered at pH 7.4 (11). When the micrographs were prepared, we were at first completely baffled. No circular bodies were seen that could represent the “blue” globules. Instead, there were numerous objects, each consisting of several concentric, crescentic stripes.

A possible explanation of these strange objects was that the “blue” globules had entirely disappeared during the preparation of the section, while another kind of cytoplasmic inclusion, totally invisible during life though large enough to be seen by the light microscope, had made its appearance. This seemed very improbable, however, because osmium tetroxide fixes phospholipids, and the crescentic objects could scarcely have avoided detection by phase-contrast, interference, and the use of vital dyes.

The second possibility was that the crescents in some unexplained way represented the “blue” globules. It occurred to me that the osmium tetroxide might have pulled the phospholipid molecules into positions more nearly parallel with one another. It is supposed that osmium tetroxide fixes unsaturated lipids of all kinds by acting at the unsaturated links of the fatty acid chains. This is borne out by the fact that osmium tetroxide does not blacken saturated fatty acids and triglycerides (11) and only reacts very slowly with synthetic saturated phospholipids (17). The work of Criese (14) suggests that osmium tetroxide oxidizes fatty acid chains at unsaturated links and inserts itself between them, thus:

\[ H - C - C = C - C - H + \frac{1}{2} O_2 + O_2O_2 = \]

The diameter of the circle of painted rods is 33 cm. In B the junction between two contiguous bimolecules has been released, and the elastic junctions between the others have pulled the membrane into a crescentic form.

Fig. 1. Photographs of a model (described in the text), illustrating hypothetically the structure of a phospholipid/water globule and its reaction to fixation by osmium tetroxide. Only one of the concentric bimolecular membranes of the globule is represented.

A is a sectional view of the intact membrane. (The diameter of the circle of painted rods is 33 cm.) In B the junction between two contiguous bimolecules has been released, and the elastic junctions between the others have pulled the membrane into a crescentic form.

Since the phospholipids that occur in nature have highly unsaturated fatty acid chains, the possibility of multiple linkages therefore presents itself.

If such links were formed, the bimolecules would be held together after fixation, not by weak intermolecular forces, but by strong covalent bonds; and the tendency of the latter would be to hold adjacent bimolecules parallel with one another. The result would be that the radius of curvature of the bimolecular layers would be increased, but little increase in the diameter of the globule would be possible. The necessary result would be the shattering of the globule along a weak line, and the production of an object that would appear in section as a group of concentric, crescentic lines, or of parallel, straight ones.

A model can be constructed to illustrate these ideas (Fig. 1). Each bimolecule is represented by a wooden rod; the hydrophil and hydrophobe parts of the molecules are painted in different colours to distinguish them. Each bimolecule is joined to the one on each side of it by two strands of elastic, fixed in such a way that they tend to bring the bimolecules nearly or quite parallel with one another. The set of wooden rods is easily formed
into a circle (Fig. 1, A), representing a section through one of the concentric membranes of a phospholipid sphere. In one place a simple device makes it possible to release two adjacent bimolecules instantaneously from one another. When the release takes place, the set of rods springs at once into a crescentic form (Fig. 1, B); or, if the outer elastic is rather tightly stretched, into a straight line. The model would probably be more perfect if a spring tended to increase slightly the distance of the central end of each bimolecule from the corresponding part of its neighbours, but this would require a more complex mechanism.

If this explanation of the origin of the crescents were true, it might be possible to find a stabilizing agent that would hold the concentric membranes in their natural form during fixation and thus cause the “blue” globules to appear as spheres or spheroids in electron micrographs. Now it has been known for a long time that phospholipid/water interfaces can be stabilized by calcium ions (22). Use is made of this fact in fixation for light-microscopy, when calcium chloride is added to formaldehyde solution (4). I therefore suggested to Mr. Chou and Dr. Meek that they should try a calcium-osmium mixture as fixative for electron-microscopy.

The micrographs of the neurones of Helix obtained with their calcium-osmium mixture fully satisfied our hopes. No crescents were seen, but only circular objects. Each of these consisted of a pale, presumably aqueous medulla and a cortex made up of several complete, concentric, osmiophil rings. Each ring could well be a transverse section of a bimolecular phospholipid membrane, for the dimensions fit this hypothesis well. Each membrane seen in the micrographs is about 6 \( \mu \) thick. It can be seen to consist of two osmiophil layers with a narrow osmiophobe layer in between. A lecithin molecule is about 2.5 \( \mu \) long (33).

A word of caution is necessary about the use of calcium chloride. We do not yet understand its mode of action in stabilizing phospholipid/water interfaces, and we cannot rely on it to act effectively in all cases. Much remains to be learnt about the role of this potentially valuable ingredient of fixative mixtures. It is hoped that those who intend to try it with other tissues will first confirm its action on the “blue” globules in the neurones of Helix, by following the technique of Chou and Meek. Bradbury and Meek (7) have already experimented with the large phospholipid droplets in the adipose cells of the leech, Glossiphonia complanata. These exhibit no definite structure when sucrose-osmium is used as a fixative, but are very clearly seen to be formed of concentric membranes when calcium-osmium is used instead.

We may perhaps be inclined to underestimate the amount of lipid that occurs in a masked form in the living cell. Bensley and Hoerr claimed long ago that the insoluble fragment of protoplasm (their “ellipsin”) contained, when dry, no less than 30% of lipid. The recent analysis of Smith and his colleagues (32) suggests that the lipid content of this material amounts to 45%. It is a curious fact that fixatives penetrate several times more slowly into tissues than into protein gels containing a similar percentage of protein (5). It seems likely that the lipid component of lipoproteins may be the barrier. Strong evidence has been brought forward to show that even chromatin contains a high proportion of lipid (8).

Different fixatives act in different ways in relation to masked lipids. Some fix them without separating lipid from protein and the former will then not be revealed by the ordinary methods for showing lipids. Other fixatives leave them in a state in which they can be split subsequently by another fluid, such as phenol (12, 13) or xylene (21); both of the constituents may then retain their solubility in suitable solvents. Other fixatives again actually dissolve lipoproteins, and the constituents of the latter (glycerophosphate, for instance) can be found in the fixative when the tissue has been removed (8). The classical work of Ciaccio (12, 13) on the unmasking of lipids by the direct action of certain fixatives has never received the attention it deserves. Some fixatives mask and fix both lipid and protein constituents; others unmask, but leave the lipid soluble in lipid-solvents.

It is particularly to be noticed that ethanol and the salts of uranium, cobalt, and cadmium have an unmasking effect (12, 13), for these substances are used in the so-called “Golgi” techniques. Golgi himself (18) used ethanol in the fixative for his “rapid” method, and salts of the three metals were used respectively by Ramón y Cajal (25), Du Fano (15), and Sowa (2) in their silver techniques. It appears that the unmasked lipids reduce silver nitrate, so that a black mark is left in the cytoplasm.

It follows from these considerations that when we see sections of parallel membranes in an electron micrograph, quite different causes may have been at work. We may perhaps be dealing with a phospholipid/water complex that existed during life in the form of a globule. Alternatively, the regular structure seen in the micrograph may have been non-existent during life.

The most satisfactory kind of electron-micrograph is one that depicts an object large enough for full study by vital and cytochemical methods. A perfect example would be provided by a visible
A fixative may fix the lipoprotein without separating the lipid from the protein (A); or it may separate the two components and fix them both (B); or it may separate them, leaving the lipid unfixed and free to be dissolved out during dehydration and embedding (C, C'); or it may dissolve the lipoprotein (D); or it may leave the lipoprotein in a state in which the separation can occur during dehydration or embedding, with solution of the lipid component (E', E').

sphere, intermediate in refractive index between lipids and ground cytoplasm, showing a dark cross between crossed nicols, responding to no cytochemical test except those for phospholipids, and represented in electron micrographs by concentric rings. There would then be perfect correspondence between vital, cytochemical, and electron-microscopical studies, and there would be every reason to suppose that the electron-micrograph gave a good representation of the sphere as it existed in the living cell. The "blue" globule approximates to this ideal.

It often happens, however, that a set of roughly parallel lines is seen in an electron micrograph, but no corresponding object is visible in life, either by phase-contrast or by the polarizing microscope. In such cases care should be taken in interpreting the micrograph. The object may perhaps be so small that if it had existed in life, it would necessarily have been invisible. Whether this is so or not, care is necessary. It is safest to regard it provisionally as a reaction-product—a body that originated by interaction between something that was present in the living cell on one hand, and one or more of the various fluids in which the tissue was subsequently soaked on the other. As Ciaccio remarked long ago (13), unmasked lipids are present in a very dispersed form during the life of the cell, but are less dispersed after unmasking. The lipid may have flowed out of a lipoprotein complex and then arranged itself in the form of a globule or strand, according to whether it was free to assume the least possible surface area, or was subjected to forces that caused distortion. If the lipid possessed hydrophil groups, there would be a tendency to the production of bimolecular membranes. Such membranes are often seen in electron micrographs in the region of the cell that is easily silvered in Golgi preparations, and these membranes may be artifacts of the kind just described.

Recent studies of the lipid components of red blood-corpuscles show that changes subsequent to the death of the cell can produce just this kind of artifact (24, 34). The unmasking is not caused by fixation in this case, but by digestive processes.
When normal corpuscles are examined by electron microscopy, nothing resembling the "blue" globule is seen; but after they have been ingested by macrophages, spheres and strands of a substance resembling myelin appear. This "myelin" is seen in electron micrographs in the form of parallel lines, representing transverse sections of membranes. Each sphere consists of a set of such membranes, arranged concentrically. The structure might appear even more clearly if a suitable fixative containing calcium ions could be devised. Polieard and his colleagues (24) interpret each concentric line as the section of a bimolecular lipid membrane. The myelin of these degenerating corpuscles shows a strong resemblance to the "blue" globules, but it must be understood that the latter exist as such during life, while the myelin arises after death, by a process of unmasking. The elaborate structure seen in the micrographs was not present when the corpuscles were alive in the blood-stream.

It is sometimes suggested that an electron micrograph can be relied on to represent the living structure, if two different fixatives give similar appearances. This is not so. The fact that different fixatives show parallel or concentric membranes in electron-micrographs does not prove that a set of parallel or concentric membranes existed in life. Any unmasking fluid may set lipids free; and if their molecules have hydrophil and hydrophobe ends, there will be a tendency for bimolecular layers to be formed.

We are apt to be led astray in the choice of our fixatives for electron microscopy. One fixative shows more detailed structure than another: how do we know that the structure was detailed in life? One fixative gives sharper contrasts than another: how do we know that the constituent parts of the object were sharply separated from one another in life?

The invention of the electron-microscope has resulted in a sudden shift of interest away from chemical composition and towards a purely morphological outlook. There is an extraordinary parallel with the events of a century ago. In the second quarter of the eighteenth century the inspiring genius of Raspail (26, 27, 28) had given birth to cytochemistry, and the new science was actively developed by Schleiden (30), von Mohl (23), and many others. The study of chemical composition proceeded as a matter of course in close association with the study of minute structure. It is an eye-opener to look at the histology books of the period and to note the chemical outlook of their authors. Henle's Allgemeine Anatomie (20), published in 1841, is a fine example. Soon afterwards, in the decade 1848-1858, the dyeing of microscopical preparations was repeatedly rediscovered (3). An immense impetus was thus given to purely morphological studies, and in the zoological field (though not in the botanical histochmistry) was knocked almost stone-dead, only to rise again in the present century. In our own time, the invention of the electron microscope has struck a very similar blow.

Another analogy with past times is provided by the history of opinion on the structure of protoplasm. The morphologists of the second half of the nineteenth century believed that their fixatives revealed the structure of living protoplasm. It required the genius of W. B. Hardy (19) to prove that they were wrong. We need another Hardy to teach a lesson to their modern counterparts.

Our inability to interpret most electron-micrographs in terms of the structure and chemical composition of the living cell shows that new, fundamental studies are required, involving the collaboration of cytochemists and electron-microscopists, or—better—the work of people who are prepared to be both cytochemists and electron-microscopists. We must work from the known towards the unknown. The most urgent necessity is a much fuller study of the appearances in electron-micrographs of known substances treated by various fixatives and embedding media. Substances of all kinds that occur in living organisms will need to be studied, but no kinds are more likely to be interesting than lipoprotein complexes.

It has been a great honour to be invited by the Histochemical Society to deliver this address. I share the honour with the past and present research workers and technicians in the Cytological Laboratory of the Department of Zoology at Oxford, and with Dr. G. Meek. New ideas generally come from free discussion among colleagues all interested in the same field of study.

I must mention also the invaluable help given to us all by Dr. M. L. Watson when we first installed our electron microscope.

SUMMARY

The purpose of the address is to point out the need for a fuller study of fixation for electron-microscopy, and especially to urge that cytochemists should interest themselves more actively in the interpretation of electron-micrographs. In some cases there is strong reason to suppose that the detailed structure seen in micrographs represents rather well the structure present in life. Certain globules in the neurones of the common
snaill, *Helix aspersa*, are carefully considered in this connection. In other cases, on the contrary, it is probable that the detailed structure seen in electron-micrographs does not represent the living condition. The structure is better interpreted as a reaction-product between the fixative used and what was present in life. In many cases, much of the detail is likely to be caused by the unmasking of hydrophilic lipids from lipoprotein complexes. Such lipids, after unmasking, would be likely to arrange themselves in double membranes. The different ways in which fixatives may react with lipoproteins are briefly described.

REFERENCES