INTERPRETATIONS OF ELECTRON MICROGRAPHS OF SINGLE AND SERIAL SECTIONS*

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(Received for publication, April 15, 1955)

The methods of thin sectioning of samples of biological material for electron microscopic observation have improved during recent years to the extent that it is now feasible to cut, mount, and examine an intact strip of serial sections (Porter and Blum, 1953; Gay and Anderson, 1954; Karrer, 1954; Williams and Kallman, 1954). The purpose of examining micrographs of serial sections is to enable one to reconstruct in three dimensions the structural details initially contained in an appreciable depth of the sectioned material. Since the observation of a single, non-serial section yields essentially two-dimensional information only, it may be anticipated that much additional insight into cellular structure can be obtained by the addition of a third dimension. When serial sections are examined, however, some apparently puzzling observations are encountered which make a complete and detailed three-dimensional reconstruction impossible. We have investigated the origins of these anomalies, and in so doing have been led into examining in some detail the more general problem of the interpretation of electron microscopic imagery of sectioned material.

Techniques for the Production and Observation of Serial Sections

It is now common experience that ribbons of serial sections can be consistently cut with ultramicrotomes designed for this purpose. Hillier and Gettner (1950) reported the production of serial sections some years ago, and Porter and Blum (1953) have demonstrated the electron micrography of a few sections cut serially. Gay and Anderson (1954) and Williams and Kallman (1954) have recently reported upon the electron micrography of serial sections whose number is limited only by the space available on the specimen grids for displaying the entire ribbon.

The primary problem in the preparation of serial sections for examination does not reside in the microtomy itself, but rather in the mounting of the ribbon of sections upon the specimen grids. We have developed a satisfactory method of preparation of serial sections which will now be described.

* This investigation was supported in part by grants from The National Foundation for Infantile Paralysis, Inc.; the National Cancer Institute of the National Institutes of Health, Public Health Service; and the American Cancer Society, Inc., upon recommendation of the Committee on Growth of the National Research Council.

Tissue-cultured HeLa cells are fixed in 1 per cent buffered osmium tetroxide (pH 7.4) for periods from 1 to 2 hours and embedded in a 90 to10 per cent mixture of butyl and methyl methacrylate. The polymerized blocks are cut with the Porter-Blum microtome with a hand-operated drive. A glass knife is used without any additional "water boat" attachment. Rather a small amount of a mixture of beeswax and rosin in equal proportions is spread across the one-quarter inch knife edge, in the form of a mound or ridge, about one-quarter inch distant from the cutting edge.[1] A few drops of water without any organic additive are placed upon the knife in the space between the wax and the cutting edge, forming a stable convex surface upon which the sections readily float. Sections are cut in the normal manner, but straight ribbons are formed only if the specimen block is carefully trimmed to a square or rectangle. In order that ten or more sections may be obtained in the form of a ribbon of sufficiently short length to be completely examined it is necessary to trim the block so that its length in the direction of cutting is 150 μ or less. Serial sections may be readily obtained, all of which are too thin to exhibit interference colors and hence appear silvery as they float on the water. Evidence to be given below indicates that none of the colorless sections is thicker than 40 mμ. Ribbons of thicker sections, appearing yellow or light copper, are even more easily obtained.

A ribbon of sections floating on water is not sufficiently motionless, nor is the hand sufficiently steady, to allow a transfer to precisely the desired area on the specimen grid. In order to improve the precision of mounting, a two-step process is used. Several circular films of formvar, stretched across lucite rings, are prepared in advance; the rings are about 5 mm. I.D., 9 mm. O.D., and 1 mm. thick. After the ribbon of sections is ferried to the center of the convex surface of the water drop, the approximate center of the formvar-filmed ring is lightly and briefly touched to the ribbon. The sections adhere to the formvar, along with a small droplet of water which may be drained away with the corner of a piece of lens tissue. The ring is best held in forceps with the formvar-filmed side down, and it should be held as parallel as possible to the plane of the floating sections when it is touched to them.

The next step is to transfer the ribbon-bearing film to the specimen grid. A grid with elongated openings is necessary if many sections are to be displayed in one continuous strip. We have used an electrodeposited grid[2] with elongated openings of about 500 $\times$ 125 μ, and also the Sjöstrand elongated grid sold by the manufacturers of Athene grids. (The material for the elongated grids should be copper, rather than nickel, inasmuch as the latter produces an asymmetry in the magnetic field of the objective lens of the microscope.) An unfilmed grid is placed on the end of a short piece of a brass rod, about 3 mm. in diameter, which is mounted vertically upward from the stage of a $\times$ 20 binocular microscope. The lucite ring is held horizontally in an attachment extending downward from the lens body of the binocular and so placed that the formvar film is in the field of view and in focus. In order that the thin sections may be seen clearly by reflected light it is desirable to have the light incident upon them parallel to the optical axis of the microscope. This is accomplished by mounting a piece of reflecting material, such as a block of aluminum, on the end of the binocular tube at an angle such that a horizontal beam from a microscope lamp will reflect vertically upon the formvar film carrying the sections. After the grid and the lucite ring are mounted, the binocular is lowered carefully. The position and orientation of the specimen grid are so adjusted that the film is caused to touch the grid with the ribbon of sections in precisely the desired location. The film is then breathed upon to enhance its attachment to the grid and the binocular lowered until the film breaks all around. The empty ring is then retracted and the filmed specimen is ready for observation.

[1] We are grateful to Dr. Alan Howatson for demonstrating this technique.

[2] Both nickel and copper grids of this nature, specified as 160/40, may be obtained from the Pyramid Diamond Drill Co. of Richmond, Virginia.

Single sections may be mounted by this two-step process, of course, and it is also possible to pick up and mount one section of a series upon one specimen grid, and the adjacent section upon another specimen grid. The latter technique allows two adjacent sections to be differently treated by chemical or physical means before observation in the microscope.

Anomalies Seen in Micrographs of Serial and Single Sections

When electron micrographs of serial sections of 40 to 50 mμ average thickness are examined in an attempt to reconstruct the detailed three-dimensional structure of the tissue being sectioned the degree of continuity seems not to be as perfect as might be anticipated. Thin sections, when examined serially, would be expected to show structural elements with a degree of continuity which would allow them to be traced in detail from one section to another, since each micrograph presumably is a two-dimensional projection of the structures throughout the section. Actually the contours of many of the larger cytoplasmic entities, such as the mitochondria, do appear to match from section to section in a general way which makes reconstruction possible, but some of these larger objects and all of the smaller ones fail to match with the expected degree of continuity. Fairly frequently there is seen a great change in the appearance of a mitochondrion, or a portion of one, in going from one section to another. As is shown in Figs. 3 and 4, one may find in two adjacent sections portions of mitochondria (large structures compared with the thickness of the section) which are distinctly different in size and shape but both of which have sharp bounding edges. Clearly, the contour of an object in the plane of the "top" surface of one section should match that in the plane of the "bottom" surface of the section immediately preceding it, but one frequently sees no matching of this nature. A striking example of the failure of thin, adjacent sections to match properly is shown in some serial sections of approximately 30 mμ thickness exhibited by Morgan *et al.* (1955, Fig. 2). Here one sees some vaccinia virus particles, approximately 250 mμ in diameter, which are sharply bounded with well defined membranes but which are present almost to full diameter in one section and either totally absent or barely present in the next section.

When attention is given to the fine linear structures, such as the double inner membranes of the mitochondria (the cristae) and other sharply delineated cytoplasmic elements, a comparison of two adjacent sections shows that the form and position of the structures change so much that they cannot be followed with certainty from one section to the other (Figs. 1 and 2). Observations such as these appear to us to require some explanation in addition to the simple one that we are looking through and seeing all the stained material throughout a planar section of appreciable thickness.

A fairly thick section ($\sim$ 60 to 80 mμ) exhibits a great variation of contrast and sharpness in its cytoplasmic structures. One commonly sees rather diffuse areas upon which appears to be superimposed some fine linear tra-

cery. There also appear sharply defined cristae in some of the mitochondria but none in other bodies which might be considered mitochondria from their general size and shape. Even in one mitochondrion seen in a single section there will appear sometimes sharp cristae in one region, and blurred, or no cristae in another region. It is important to know whether these variations are due to the lack of cristae in some mitochondria, or to a variation in fixation over a single cell, or whether they may be explained on a geometrical basis.

Several investigators (Palade, 1952; Rhodin, 1954; Sjöstrand, 1953 *a*) have studied the structure and spacing of the cristae found in the mitochondria of most cells. Rhodin (1954) has measured the thickness and separation of the double membranes of several hundred cristae and has reported an average thickness of 5.5 mμ and a center-to-center separation of 12.9 mμ, the latter being constant within 5 per cent. The constancy of the intermembrane separation is surprising in consideration of the randomness of the angles at which the mitochondria must have been cut. A transection other than in a plane perpendicular to the plane of the double membranes should cause each of them to appear broader, should increase their center-to-center separation, and should decrease the separation between their inner edges. For example, if the section is 20 mμ thick, and the transection angle is 45° from the normal to the plane of the cristae, each member should appear at least 14 mμ thick owing to the geometrical factor alone.

Another puzzling feature of micrographs of sections is best seen in tissues containing well developed virus particles of large size such as influenza, vaccinia, (Morgan *et al.* 1954), and meningopneumonitis (Fig. 9). One sees occasional particles which are well formed, with a sharp boundary over part of their periphery, but the remainder of the periphery is apparently missing. As Fig. 9 shows, this effect is to be associated with the microtomy of the section, since the missing portion of the particle is usually along an edge parallel to the knife edge. The presence of these bizarre forms raises the following questions: (1) is the missing portion of the virus initially absent (or unstained) in the embedded material, (2) is the effect due to the geometry of the microscopic observation, or (3) is the portion of the virus particle gouged out of the section during microtomy?

OBSERVATIONS

In an attempt to find the answers to the above described anomalies we have carried out experiments of the following nature: (*a*) adjacent sections mounted in three ways with respect to the order of cutting of the two faces of each section, (*b*) high-angle stereoscopy of the same and adjacent sections, (*c*) observations of sections which have been shadowed prior to and subsequent to exposure to the electron beam of the microscope.

(*a*) *Observation of Adjacent Sections.*—A generalized hypothesis that would seem to aid in explaining some of the anomalies encountered in the interpretation of micrographs of single and serial sections is that we obtain information about structural detail from only a thin portion, Δt, of each section of thickness t. In particular this might explain the failure of serial sections to have three-dimensional continuity. Our first hypothesis was that only at the free surface of each section (the surface away from the formvar film) was there sufficient electron contrast to allow structural detail to be exhibited. This hypothesis can be tested by mounting adjacent sections in different ways and observing the effect upon the degree of matching of the sections.

When a section is cut it has two surfaces which are different in respect to the order in which they are formed. The surfaces formed first and second we will call A and B; the B surface is the one in contact with the water as the section floats away from the knife edge. Two adjacent sections (which we will number 1 and 2 in the order of cutting) are usually mounted upon the microscope grid with the surfaces 1 B and 2 B away from the formvar, when the two-step mounting method is used. They may also be mounted with the 1 A and 2 B surfaces or with the 1 B and 2 A surfaces away from the formvar by appropriate manipulation prior to transfer to the formvar-coated rings. Surfaces 1 B and 2 A should be identical inasmuch as they are two sides of the same cut. If our hypothesis is correct, the 1 B–2 A mounting should exhibit better matching than the 1 A–2 B mounting.

Many pairs of adjacent sections have been mounted in the three ways just described. Fairly thick sections, 50 to 80 mμ, were used in order to accentuate any differential effect. The results are somewhat obscured by the variability of structural detail in the HeLa cell tissues, but the general conclusion drawn from the work is that the hypothesis is inadequate. No systematic difference exists in the degree of matching obtainable from adjacent sections mounted in the three ways described.

Individual sections have also been examined when placed in the electron microscope with formvar toward and away from the objective pole-piece. Critical examination of the micrographs fails to reveal any differences either in sharpness or in form of the fine structure observed. One must conclude, then, that whatever is seen in a section is observed equally well regardless of the orientation of the mounting, as long as the electron beam penetrates the section normal to its plane.

(*b*) *Stereoscopy.*—Stereoscopy seems to have been seldom, if ever, applied to the observation of sections, probably because the normal stereoholder employed in the electron microscope would not be expected to afford any stereorelief. We have made a 60° included-angle stereoholder by cutting the end-surface of a standard holder at an angle of 30° from its initial plane. A corresponding specimen grid cap is made with a 30° surface within it. Stereo-

scopy is accomplished by rotating the specimen grid (but not the cap) through 180° in its own plane between the two exposures in the electron microscope. If a section of, say, 80 mμ thickness is micrographed, two stereopictures taken with the 60° holder should show an 80 mμ displacement between the two surfaces of the section—a displacement observable at a magnification of 50,000.

We have found that thick (~ 80 to 120 mμ) sections examined by stereoscopy exhibit some three-dimensional relief. Certain small structures can apparently be followed through a section, although the stereorelief is so small that interpretation is somewhat uncertain. A striking feature seen in the stereopairs is that the sharply detailed structures of the cytoplasmic components are usually non-identical in the two micrographs. For example, one of the small (~ 40 mμ) circularly outlined structures seen abundantly in a thick section may appear in one micrograph to be very sharply outlined over part of its periphery and poorly outlined elsewhere. In the stereomate the peripheral regions of sharp delineation will be complementary to those seen in the first picture.

The mitochondrial cristae exhibit a similar effect. One picture of a stereopair may show a sharply outlined set of parallel double membranes, while the other picture may show them as indistinct or indiscernible. This apparent alteration in structure is occasionally seen within a single mitochondrion (Figs. 5 and 6). Evidently, as others have surmised (Bradfield, 1953; Morgan *et al.*, 1954) the dependence of apparent sharpness of membranous structures upon angle of incidence of the electrons is quite marked. This observation leads one to conclude that only those mitochondria which have been transected in a direction almost normal to the plane of their cristae will appear to contain them, and that many objects grossly like mitochondria may appear to be devoid of cristae despite their actual presence. A single section observed in the usual manner may well contain many more sharp, linear structures than are seen. The apparent absence of such structures within any cytoplasmic particulate cannot be taken as proof that they are really absent or that the staining and fixation are inadequate.

The results obtained by stereoscopy serve to explain the constancy of the intramembrane separation of mitochondrial cristae reported by Rhodin (1954) and discussed earlier in this paper. Since only the sharpest appearing membranes can be measured to the very small dimension reported, it is evident that only the membranes transected perpendicularly to their planes could have been measured. This inadvertent selection eliminates the spread of intramembrane separations which would be anticipated from the geometrical considerations discussed earlier.

(*c*) *Determination of Thickness of Sections.*—The thickness of sections can be estimated by observation of their interference colors while they are floating

on water, if they are thick enough to exhibit any color. Sections thinner than about 50 mμ appear colorless, but their thickness may be estimated, although quite inexactly, by noting the intensity of their silvery reflection. Stereoscopy, and shadowing (Porter and Blum, 1953; Sjöstrand, 1953 *b*), provide other means of measuring section thickness, and may be used to calibrate the eye in judging thickness by the color and intensity of light reflected by the section. Stereoscopy can be employed if some sharply delineated objects can be formed (or placed) in the plane of the top surface of the section and in the plane of the formvar below. If it is assumed that the relative position of the edges of the two sharply bounded objects may be measured within 5 mμ, stereoscopy with a 60° angle would allow section thicknesses to be measured to 5 mμ.

We have employed uranium shadowing as a means of exhibiting objects of high contrast with which to measure stereodisplacements. The section is heavily shadowed before it is observed in the microscope and a 60° stereo pair of micrographs is obtained of a region near the edge of the section. On each electron micrograph of the stereopair two small, sharply defined irregularities (made visible by the shadowing) are chosen, one on the surface of the section near an edge, and one on the surface of the formvar. The distance between the chosen points is measured on both micrographs, in a direction perpendicular to the axis of stereotilt, and from the relative displacement of the points the thickness of the section is calculated. It is estimated that the section thickness can be measured within 10 mμ by this method.

The thickness of the same section used in the above stereomethod can be calculated by measuring on another micrograph the length of the shadow cast by the edge. This calculation can be made if the shadows have been formed with their length perpendicular to the edge of the section, if the local shadow angle is precisely known, and if the micrograph is obtained with the specimen mounted in a conventional holder. The disadvantages of this method and of the stereomethod are that the thickness is measured only near the edge of the section and that the section so preshadowed is unfit for showing any internal structure.

Measurements of thickness by the two methods just described consistently yield two different values for the same section. The stereo thickness is about one-half the shadow thickness; *i.e.*, the same portion of a section measured to be 100 mμ thick by the width of the shadow formed by its edge is found to be about 50 mμ thick by stereoscopy. The origin of this discrepancy is discussed in the following.

(*d*) *Sublimation of Sections.*—The reason for the discrepancy in section thickness as measured by the two methods just described is that the shadowing method measures the thickness *before* the electron microscopy has been performed while the stereomethod measures it *afterward.* If any recession

of the surface takes place during the microscopy its effect will be noticed only by means of stereoscopy, since the shadowing has left "footprints" of the section as cut. As Watson (1953) and Hillier and Cannan (1951) have shown, the electron beam may be used for deliberate removal of the polymer from the sectioned tissue. No quantitative investigation seems to have been made, however, of the sublimation effect of the exposure to the beam during normal microscopy.

We have investigated the degree of sublimation of sectioned material undergone during ordinary microscopy by three methods and find that the results are quantitatively consistent. Figs. 7 and 8 show a matched pair of pictures of a section (initially about 50 mμ thick) micrographed first in the normal manner and then remicrographed after shadowing with uranium. The microscopy was performed with an objective aperture of 25 μ diameter, and a condenser aperture of 0.4 mm. diameter, and with an electron illumination just adequate to provide a proper exposure in 5 seconds at a magnification of 6,000. As can be seen there is evidence of a considerable amount of recession of the polymer owing to sublimation. The raised structures in the tissues do not appear in sharp relief, but seem to be obscured by a mounded overlay of material. It would be of interest to know whether some of the tissue sublimes, or whether it shrinks toward the polymer surface as sublimation proceeds, but there seems to be no way of ascertaining this. Shadow lengths can be only poorly measured from such rounded material, but we believe that the tissue material has shrunk somewhat, since it does not project out of the sublimed polymer surface as far as that surface has receded from its initial level.

The observation of the same section both before and after shadowing is of use in elucidating the anomaly, mentioned earlier, in which a large virus particle is seen in good contrast over part of its periphery and very indistinctly over the remainder. Figs. 9 and 10 show this effect for the virus particles of meningopneumonitis.[3] We have established by stereoscopy that this effect is not due to differing orientations of the virus peripheral membrane with respect to the transection plane, since two stereomicrographs show the same variation in transparency in any one sectioned virus particle. The question then arises as to whether the more transparent region represents a relatively thinner portion of the section or whether it represents a region of the virus where the staining has been relatively poor. As Fig. 10 shows the sectioned virus body is actually thinner in the region where it appears least dense in Fig. 9.

The change in thickness of a section resulting from normal exposure to the electron beam can also be measured by shadowing the edge of a section

[3] The growth of this virus in HeLa cells is under study by sectioning methods as part of a collaborative program with Dr. T. T. Crocker.

both before and after exposure in the microscope. When this is done the results are in accord with the stereoscopic observations: the section recedes to about half its initial thickness during the electron micrography. The sublimation appears to be quite rapid; at least we have been unable to secure a good micrograph in a time short enough to prevent the recession of the surface by sublimation.

A third method of estimating the change in section thickness brought about by sublimation is simply to inspect the section by reflected light before and after exposure to the electron beam. This method also offers some assurance that the thickness as measured only at the edge is a satisfactory representation of the entire section. A semithick section after transfer to the formvar membrane will appear copper-colored when examined by specular reflection with the light at 45° incidence. After it has been exposed to the electron beam it will appear either silvery or light straw-yellow, a reliable indication that either its thickness or density, or both, have decreased. The evidence gained by shadowing, stereoscopy, and interference colors shows with some degree of quantitative consistency that at least the polymer in sections is reduced to about one-half its thickness by the time an electron micrograph is taken.

Attempts have been made to see whether sandwiching a section between two films of formvar would change appreciably its electron microscopic appearance or its susceptibility to sublimation. The formvar film, with the section on its upper surface, is first mounted on the specimen grid in the manner described earlier, and another film of formvar is then lowered over the grid. Electron micrographs of such a specimen, compared with micrographs of an adjacent serial section, not sandwiched, indicate that the contrast and resolution are very slightly decreased by the overlying formvar film. The effect of this film upon the sublimation of the polymer may be estimated by examining the interference colors before and after exposure in the microscope. Our conclusion is that the overlying film has negligible effect in protecting the polymer from sublimation. This observation has bearing upon the postexposure shadowing experiments mentioned above. Since the overlying film is seen to have little effect upon the sublimation rate, it may be expected that in the exposure of a normally mounted specimen to the electron beam some of the observed decrease in section thickness is due to sublimation at the polymer-formvar interface. Consequently, the free surface has not undergone all the sublimation, and the elevation of the unsublimed tissue material (measured by shadow lengths) would not be as great as the total decrease in thickness of the section.

(*e*) *Preshadowed Surfaces of Sections.*—The failure we have experienced in attempting to improve the continuity of structures seen in two serial sections by mounting them in alternate ways, as described in (*a*) above, has

led us to investigate the possibility that even two adjacent cut *surfaces* (the 1 B and 2 A surfaces) might fail to match in profile; *i.e.*, fail to match as a die and its impression. If the operation of "cutting" a section is in fact a ripping or gouging action (Hillier and Gettner, 1950) with some removal of material, the two surfaces created at one stroke of the microtome would not be expected to match in their surface detail. This possibility can be investigated by mounting two sections with the 1 B and 2 A sides uppermost and shadowing very heavily with uranium to accentuate surface detail and obscure inner structures. Observations involving preshadowing have been made on vesicular regions of HeLa cells containing many particles of the meningopneumonitis virus, and on normal appearing regions. The constancy of the interference colors of sections before and after shadowing, as well as the sharpness of the shadows of the edges of the sections, demonstrates that any sublimation which might occur during shadowing is negligible compared with that occurring during the microscopy.

The most noticeable aspect of the micrographs of the preshadowed vesicular regions containing virus particles (Fig. 13) is that every particle appears indented (like a thumb mark in clay) and that the membranes surrounding the particles appear sharply recessed into the polymer. This appearance is universal whether one observes a 1 B or 2 A surface. There is no profile matching between these surfaces: a region which appears recessed in one does not appear elevated in the other.

The results of preshadowing the 1 B and 2 A surfaces of sections of normal regions of HeLa cells are exemplified in Figs. 11 and 12. Membranous structures, such as the peripheral membranes of mitochondria and nuclei, appear sharply indented in both sections. More important, some of the larger structures fail to appear to be of the same size and shape. The failure to match varies in degree from section to section and from place to place in the same section.

The evidence gained from these preshadowing experiments substantiates the notion that there is some removal of material between sections. If the cutting action were only a tearing one the depressed areas in the 1 B surface would be elevated in the 2 A surface. The amount of removed material must vary over the section surface, ranging from the great amount obviously removed in the gross knife marks (gouges) to a probably imperceptible amount where the knife is truly sharp. The average magnitude of the effect, and the eventual location of the material removed, are both unknown.

If material is bodily removed between sections, the observation that portions of large virus particles are apparently missing in sections becomes understandable. The fixed virus body probably has rheological properties different from the surrounding polymer, and when cutting occurs there will be portions of the virus body wholly removed from the section.

The preshadowing referred to above was performed with the shadowing direction perpendicular to the direction of cutting. When the direction of shadowing is along the direction of cutting, the surface of the section appears as an array of parallel wrinkles, with an average spacing of about 30 mμ (Fig. 14). This effect is doubtless the real expression of the so called compression of sections. They cannot be truly compressed, since methacrylate polymer is almost incompressible, though elastic. Nor is there a kind of distortion obeying Poisson's ratio, since cut sections are no wider than the block from which they are cut. It appears that the "compression" is really a wrinkling or rippling of the section, resulting in a decrease of length in the cutting direction and hence an increase in the average thickness of the section. If there is no genuine distortion obeying Poisson's ratio, *the length of the major axis of the ellipse resulting from the sectioning of a sphere, as in the case of viruses, is to be taken as the correct diameter of the initially spherical particle.*

DISCUSSION

Although the work reported here has not resulted in definitive conclusions, enough has been found to engender caution in the purely physical and geometrical aspects of the interpretation of micrographs of sections.

The contrast exhibited by membrane-like structures in cells is severely affected by the orientation of the membranes with respect to the direction of the electron beam. As a consequence, one sees less structural detail in any one micrograph than actually exists in the section. High-angle stereoscopic micrographs of the same section help in exhibiting fine structure otherwise missed. The appearance of cristae in mitochondrion-like bodies may be taken as sufficient evidence that the bodies are indeed mitochondria but may not be taken as a necessary condition for identification.

Serial sections do not appear to present information concerning fine structures in a manner as continuous as anticipated. There seem to be two reasons for the evident discontinuity. Sublimation of the section in the electron microscope is so rapid and is of sufficient magnitude that it is safe to conclude that a section is never micrographed as cut. Rather, what is micrographed is a central plane, probably undisturbed, and bounded on each side by a plane from which much of the polymer has been sublimed. Secondly, even the serial sections as cut do not appear to represent a continuity of the material existing within the polymerized block. Considerable gouging evidently takes place when the sectioned slice is separated by a glass knife from the block, resulting in a removal of material. The action of cutting would be at best a tearing, or parting, even with a perfect knife edge. But it is reasonable to believe that a knife edge created by causing two breaks in a piece of glass to intersect will consist of a linear array of imperfections of various sizes, with some of them in a range of size comparable with the thickness of a thin

section. Such imperfections would very likely take the form of small, conchoidal, chipped out regions. In such a region the effective included angle of the cutting "edge" would not be 45° (the over-all included angle of the knife) but would be more nearly 90°. It is not surprising to anticipate that cutting with such a blunt angle would result in bodily removal of material of a thickness comparable with the widths of the imperfections. Where this effect is severe the results can easily be seen as linear, thin regions in the serial sections as they float on the water, or as observed in the microscope. Only if material is removed can there be a thin area in the same position in several adjacent sections, as is commonly observed in serial sections. These areas are generally avoided in microscopy, but there is no reason to believe such knife marks are all-or-none phenomena. It is more reasonable to expect that those regions of the section which appear non-marked will differ only in degree from the obviously marked ones.

The observations and conclusions reported here, as well as the published observations of others, strongly indicate that at the present time serial sections do not furnish information which is adequate for three-dimensional reconstruction of *detailed* cellular structures. For example, it appears fruitless to attempt to draw conclusions regarding the three-dimensional convolutions of the cristae within mitochondria, since in micrographs of serial sections it will frequently occur that a given crista cannot be unambiguously followed from one section to another. This circumstance raises a more general question which has not existed prior to the availability of serial sections for careful examination. In a single section structures are found which exhibit extremely fine detail, and considerable effort has been spent in attempting to obtain higher effective resolution down to the range of 1 mμ (Flewett and Eaves, 1954). In a single section, however, there is no way to assess the degree to which the observed ultrafine structures have been influenced and perhaps distorted by the effect of microtomy and microscopy. In other words, there is no control possible; one cannot remove the same section over again from the block to see whether a micrograph of a recut section would be identical with the one obtained initially. The best one can do to assess the credibility of observed detailed structures is to examine thin, adjacent sections to see whether the detailed structures are sufficiently similar to be compatible with the thickness of material separating the midplane of the sections. The degree of compatibility does not seem sufficient to justify belief that, aside from the gross effects of "compression," there is always a one-to-one correspondence between the relative positions of detailed structural elements in a given volume of the block and the positions which these elements have when this volume is converted to a section.

If the advantages inherent in the observation of serial sections are to be fully exploited it would seem that a necessary development is the improve-

ment of the cutting edge of the knife. At present there is no way of gauging the excellence of any knife edge except by comparing the appearance of a section with what we imagine to be the appearance of a perfectly cut section. Optical examination at a magnification of 1000 or so will allow the rejection of grossly inferior knife edges, but yields no direct information on the scale of size of the thickness of a section. It would be useful if small objects were available which were of known size after embedding, and which would have the general cutting characteristics of tissue. Such objects could be used to evaluate the degree of distortion occurring during microtomy and the degree to which embedded material is removed between adjacent sections.

SUMMARY

A method of securing serial sections for electron microscopy is described. Serial sections present certain anomalies of interpretation of a nature such that a complete and detailed three-dimensional reconstruction of the sectioned tissue cannot be made. These anomalies are discussed, as well as those which have been encountered in the interpretation of single sections. Observations of the following kinds have been made in an attempt to elucidate the interpretation of single and serial sections: differing methods of mounting adjacent sections, observation of the same section by high-angle stereoscopy, and examination of sections which have been shadowed prior to and subsequent to electron microscopy.

It is found that the appearance of sections is independent of the choice of side to be placed against the formvar films. Stereoscopy shows that the appearance of fine structures is strongly dependent upon the direction of the penetrating electron beam with respect to the plane of the structures. Stereoscopy, combined with shadowing, shows quantitatively that extensive sublimation of polymer occurs upon normal exposure in the electron microscope. Observation of sections shadowed prior to electron microscopy indicates that varying amounts of material are removed between sections by the action of microtomy; *i.e.*, it is probable that the sum of the thicknesses of several serial sections is considerably less than the total thickness of material removed from the block. It is believed that this effect, combined with the effect of sublimation, aids in explaining the failure of adjacent sections to exhibit continuity in their detailed structures.

The authors gratefully acknowledge the excellent technical assistance of Mr. Joseph Toby in the preparation of the photographs for this paper.

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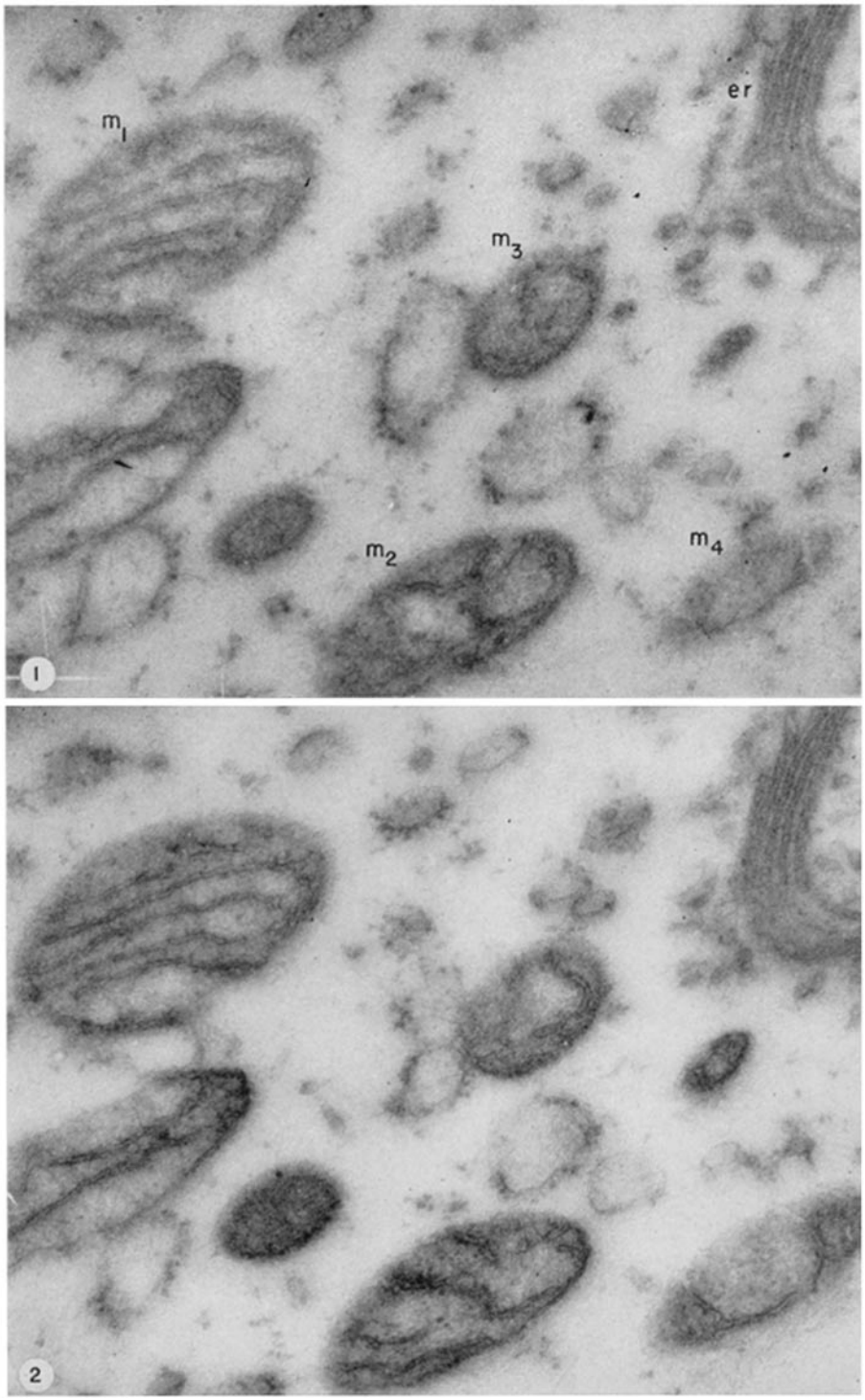
EXPLANATION OF PLATES

PLATE 87

FIGS. 1 and 2. Electron micrographs of two consecutive serial sections of a cytoplasmic region of a HeLa cell. These micrographs serve to illustrate the degree to which the fine structures of cellular components appear to vary in form from one section to another. The thickness of each section is estimated to be 20 to 30 mμ. The components marked $m_1 \ldots m_4$ are mitochondria, and the set of parallel membranes marked *er* is presumably a portion of the endoplasmic reticulum. It can be seen in m_2, as an example, that double membraned cristae are present in each section, but it is impossible to follow the form of the cristae from Fig. 1 to Fig. 2. Close inspection of the other mitochondria shows that this difficulty is commonly encountered. The parallel lamellae, *er*, are readily identifiable from one section to the other, indicating that the parallelism extends through a large range of thickness in the cell. Scattered throughout the cytoplasm are the small, sharply bounded structures of the endoplasmic reticulum which change more in size and shape than would be expected in two thin adjacent sections. At this magnification, the lateral dimension corresponding to the section thickness is only 2 mm. × 80,000.

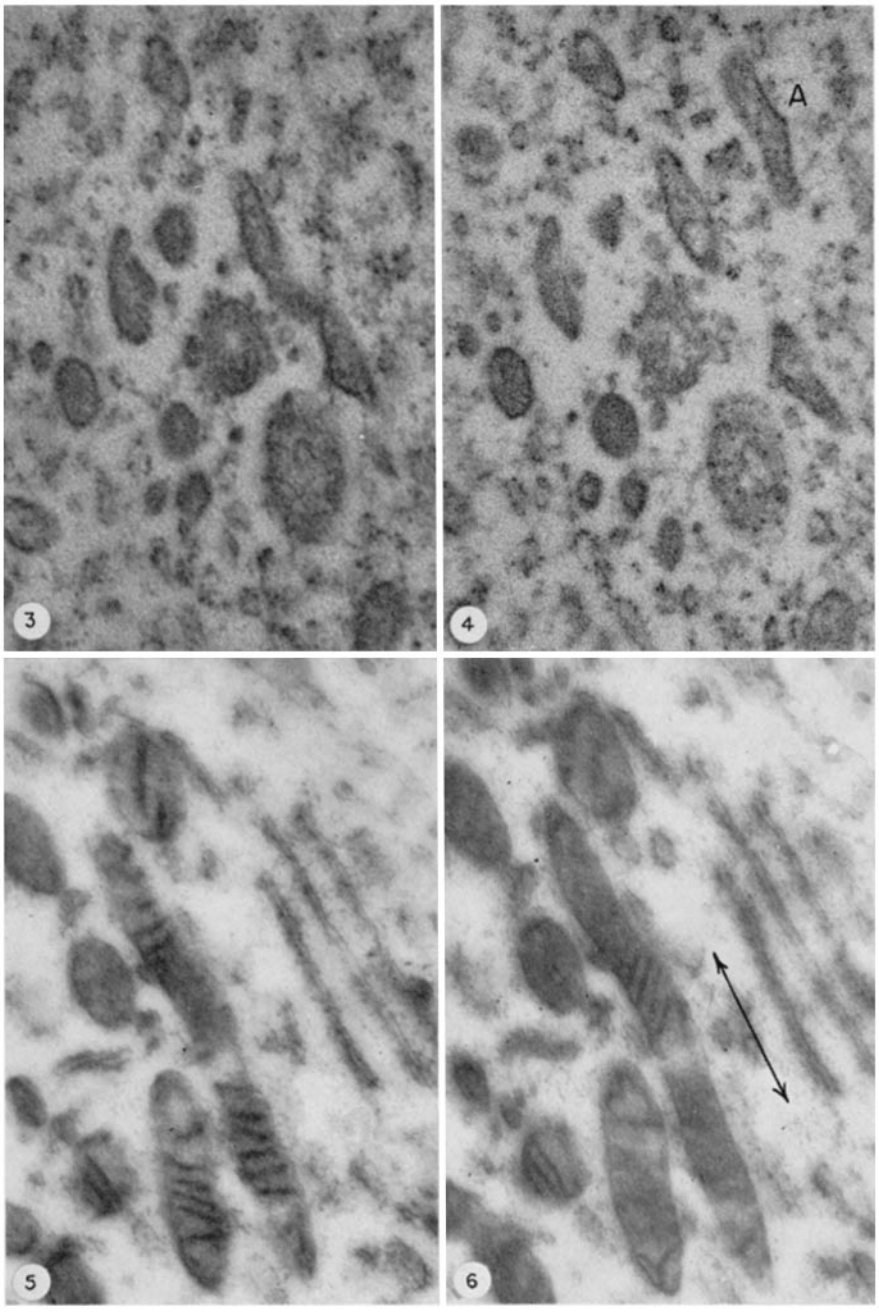


(Williams and Kallman: Interpretations of electron micrographs of sections)

PLATE 88

FIGS. 3 and 4. Electron micrographs of two adjacent sections, approximately 40 mμ thick, showing a region of the cytoplasm of a HeLa cell. Although a general sort of continuity is present from one section to another, careful inspection shows that most of the smaller structures cannot be followed from one section to the other. The object shown at *A* has a length of 700 mμ and a width of 170 mμ in Fig. 4, but only the faintest sign of its presence is apparent in Fig. 3. While this is an extreme example of two adjacent serial sections failing to exhibit the anticipated continuity of structures, inspection of the smaller objects discloses that this failure is fairly general. × 30,000.

FIGS. 5 and 6. A stereoscopic pair of electron micrographs of a small region of the cytoplasm of a HeLa cell. The two micrographs were secured from a single section which was tilted 60° between exposures. The direction of stereoscopic tilt is shown by the arrows. Several mitochondria, and portions of mitochondria, are shown. It is evident that the appearance of the cristae is affected by the angle at which the electron beam penetrates the specimen, since there are very few cristae common to the two micrographs. Those that are in common are oriented with their length approximately parallel to the direction of stereotilt. × 36,000.

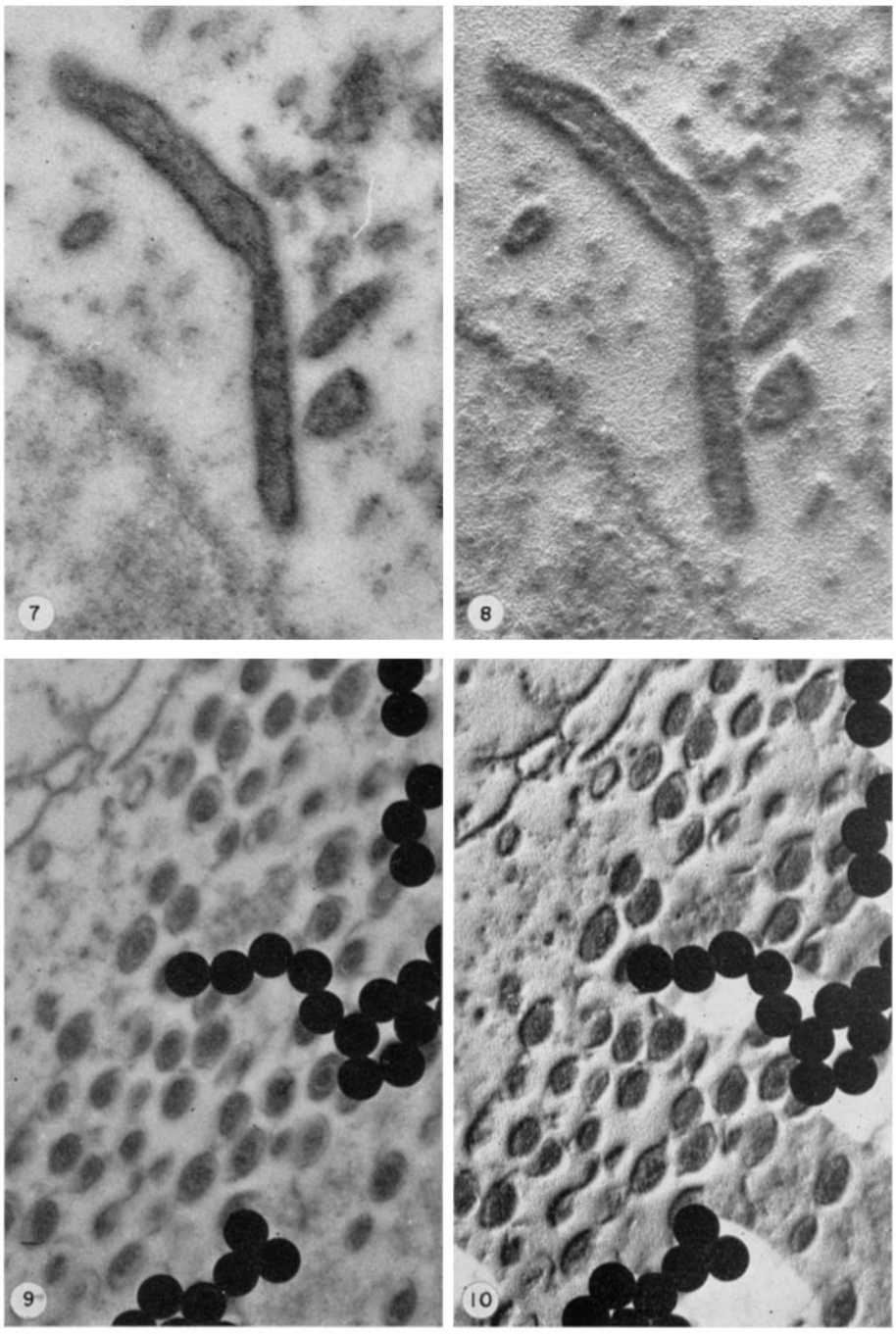


(Williams and Kallman: Interpretations of electron micrographs of sections)

PLATE 89

FIGS. 7 and 8. Electron micrographs of the same section of a HeLa cell: Fig. 7 is a normal micrograph, while Fig. 8 is one taken subsequently and after shadowing. In this photograph, and in all the succeeding ones of shadowed specimens, the print is a direct positive with shadows appearing light. The effect of partial sublimation of the polymer is particularly evidenced by the shadows cast by the nuclear membrane and by the mitochondria. The local shadow angle is $\tan^{-1} 0.50$. It is estimated that this section was initially 50 mμ thick and that the average thickness of the polymer after sublimation is 25 mμ. The exposed elements of tissue appear to be partially embedded in unsublimed polymer. For this reason there is uncertainty as to whether or not the tissue has receded somewhat or has been distorted by the sublimation. It is to be emphasized that the shadowing serves only to exhibit the nonplanar detail already present. Hills and ridges like those seen in Fig. 8 actually exist on the surface of all similar sections as they are normally micrographed without shadowing. × 20,000.

FIGS. 9 and 10. Two electron micrographs of the same section of a vesicular region of a HeLa cell containing elementary bodies of meningopneumonitis. The opaque spheres are polystyrene latex spheres sprayed (in excess) upon the section to facilitate technical evaluation of the micrograph. Fig. 9 is a normal electron micrograph showing the elementary bodies with their usual elliptical shape due to wrinkling of the section during microtomy. Several of these bodies are seen to have peripheries which are only in part complete and opaque (for example, the particle in the lower right hand corner). Fig. 10 is the same section micrographed after shadowing. It is seen that the relatively opaque and transparent portions of the particles in Fig. 9 are represented in Fig. 10 by more or less elevated regions. This relationship demonstrates that the variation of opacity of the particles in the unshadowed micrograph can be accounted for by local variations in the thickness of the cut particles. × 23,000.



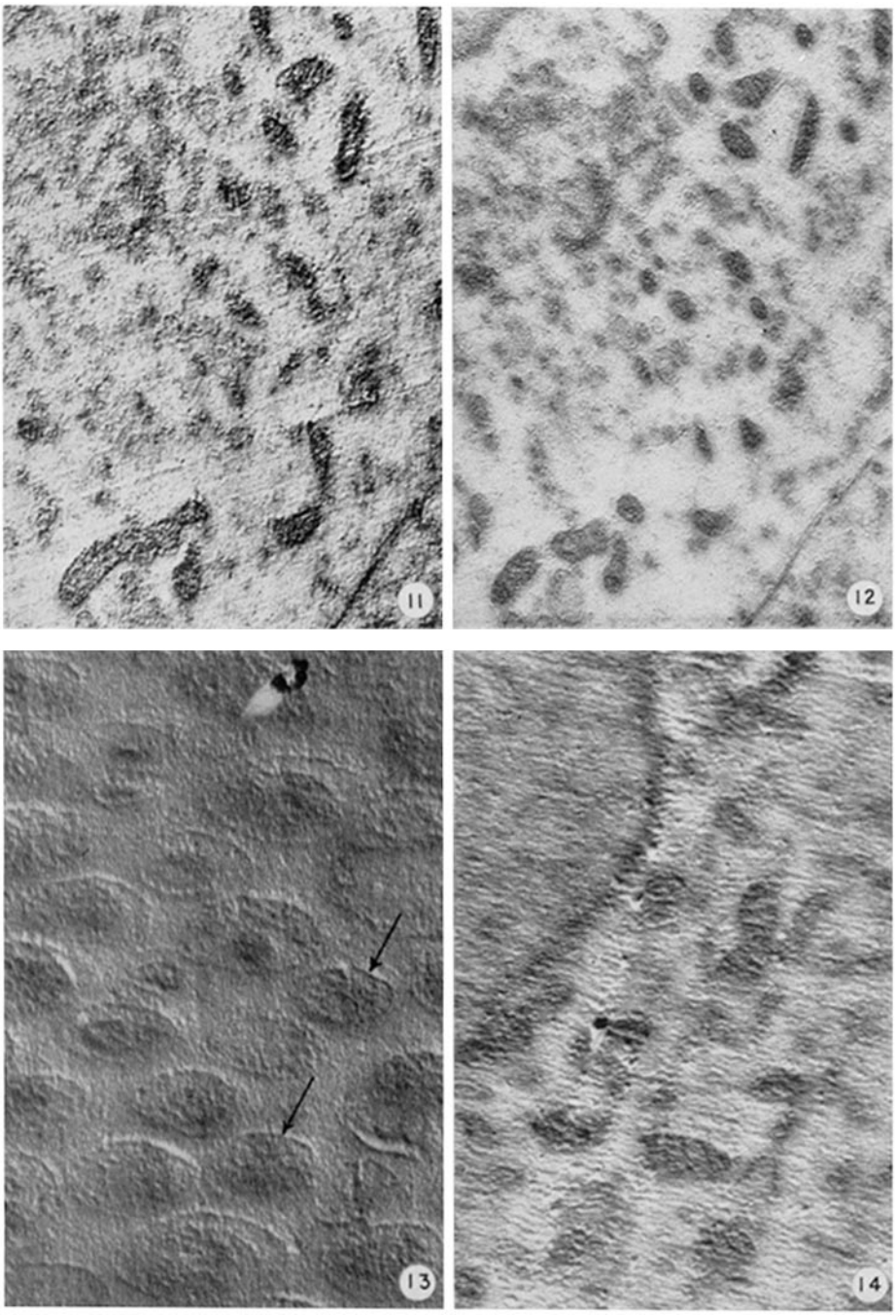
(Williams and Kallman: Interpretations of electron micrographs of sections)

PLATE 90

FIGS. 11 and 12. Electron micrographs of the two adjacent sections of a portion of a HeLa cell. The first section (Fig. 11) was mounted with its 1 B surface uppermost; the second (Fig. 12) was mounted with its 2 A surface uppermost (see text). Both sections were *heavily* shadowed with uranium prior to microscopy, and with the shadowing direction perpendicular to the direction of cutting. The 1 B and 2 A surfaces were created simultaneously by the microtomy. If the sectioning were accomplished by an ideal cutting or tearing action it might be expected that the surface appearance of the two sections would be identical except for inversion of any elevation or depression. It can be seen that the exposed surfaces of the mitochondria are brought into relief by the shadowing, and that the shadowed contours of some of them fail to match in the two micrographs. This observation can be explained by assuming that some material has been removed from between the two surfaces; *i.e.*, that they are independent surfaces, though created simultaneously. It is to be noted that the knife marks show on these micrographs, but do not appear on postshadowed ones, such as Figs. 8 and 10. In the latter cases the marks are evidently erased by sublimation. × 15,000.

FIG. 13. An electron micrograph of a group of elementary bodies of meningopneumonitis found in a portion of an infected HeLa cell. The section was mounted with its A surface (see text) uppermost and was heavily shadowed prior to microscopy. The direction of shadowing is indicated by the shadow cast by the small object at the top of the micrograph. From a knowledge of the shadow direction it can be ascertained that the surfaces of the virus bodies are depressions in the section surface, and that their peripheral membranes are sharply recessed. Two examples of recessed membranes are shown by arrows. (A persistent optical illusion causes the membranes frequently to appear raised. Observation of the micrograph at different orientations helps to reveal their true recessed nature.) Although the B surface of the preceding section is not shown here, its appearance is similar to that in this micrograph, with the virus surfaces depressed and the membranes recessed. × 70,000.

FIG. 14. An electron micrograph of a HeLa cell section which was shadowed *parallel* to the direction of cutting prior to microscopy. The surface is seen to consist of parallel, approximately equidistant ripples only about 30 mμ apart. These are not to be confused with the gross wrinkles observed in sections as they float on the water, nor with the "chatter" marks occasionally seen in sections. The ripples shown here are ubiquitous, are on a very fine scale, and disappear by sublimation during microscopy (as shown by postshadowing), while chatter marks are only slightly sublimed away during microscopy. The fine ripples are an expression of the gross "compression" encountered during microtomy, in which a section is never found to have as large an area as the face of the block from which it comes. There appears to be no measurable lateral expansion of a section during cutting, and hence dimensions measured perpendicularly to the cutting direction are probably unaffected by microtomy. × 30,000.



(Williams and Kallman: Interpretations of electron micrographs of sections)