is difficult quantitatively to compare the growth of rickettsiae in dead embryos with that in living ones, as several factors obviously influence the final result. The maximum number of rickettsiae obtainable per egg is perhaps greater in eggs containing living embryos, since their yolk sacs are more extensive than those of eggs which have developed for only 3 days. However, the concentration of rickettsiae per cell in the infected cells of dead embryos may surpass that reached in living ones.

The growth period for rickettsiae in dead embryos may easily be extended to 16 days, or double the time customary in work with living embryos. Therefore even minute inocula have a chance to multiply significantly. This was determined in the following manner: 3-day-old dead embryos and 7-day-old living embryos were inoculated in parallel series with decreasing amounts of the same suspension of rickettsiae. Inocula too small to yield a positive smear after the usual 7-days' incubation in the living embryos yielded smears containing numerous rickettsiae after 14 days' incubation in the dead embryos.

Several advantages are inherent in our method. Eggs have to be incubated prior to use for only 3 days, and may then be stored for sometime at room temperature like ordinary culture media. This fact is especially convenient in field work, in which eggs may be inoculated on the spot and thereafter transported to the laboratory without particular regard to correct incubation temperature, humidity, or protection from vibration during transport, as is necessary when dealing with living eggs. Furthermore, this method is economical, since the percentage of eggs which have to be discarded on account of premature death of the embryo is, of course, considerably reduced.

Summary. 1. Chick embryos which were killed by chilling on the 3rd day of development and were thereafter maintained at 37°C for 16 days still contained living cells.

2. *Rickettsia prowazeki* multiplied abundantly in dead chick embryos which contained surviving cells.

3. The significance of these findings and some advantages inherent in this method are discussed.

16344

Sectioning Techniques for Electron Microscopy Using a Conventional Microtome.

DANIEL C. PEASE AND RICHARD F. BAKER.

From the Departments of Anatomy and Experimental Medicine, School of Medicine, The University of Southern California, Los Angeles, Calif.

Sectioning biological material for use with the electron microscope poses special problems. Useful thicknesses can be measured only in fractions of a micron so that cutting becomes a major difficulty. Sections have to be examined *in vacuo,* so that the mode of drying is a matter for serious consideration.

The authors know of only 3 prior attempts to adapt more or less conventional histological techniques to the preparation of specimens for the electron microscope. Richards, Anderson and Hance[1] took embedded material and faced the block in a standard microtome. They then changed the angle of the block and cut wedges which they hoped would taper into the fractional micron range. To a certain extent they were successful, but apparently were unable to find a completely satisfactory solution to embedding and mounting problems. Von Ardenne[2] employed essentially the same principle. Sjöstrand[3] has also attempted

[1] Richards, G. A., Anderson, T. F., and Hance, R. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 148.

to cut thin sections in an unspecified manner. Interest then shifted to the development of high speed microtomes by O'Brien and McKinley[4] and Fullam and Gessler.[5] Their cutting edges, moving with tremendous linear speeds, have cut sufficiently thin sections for effective use. But the design and construction of the high speed microtome is a complex engineering problem, and it is certain that it will always be a very costly instrument, not usually available.

The possibility that standard histological techniques could be adapted was first suggested to the authors by Dr. F. Kiss of Hungary. In conversations he stated that he had often cut fractional micron sections for use with the light microscope. He implied that the size of the block was a critical factor, and that the material should be doubly embedded in collodion and paraffin. It now appears that his claim was fully justified, for we have consistently been able to cut sections 0.2 microns in thickness.

A Spencer rotary microtome model No. 820 is used, but altered simply so that the unit of advance is approximately 1/10 of the calibrated value. To accomplish this the angle of lever action is reduced 90% with the attachment shown in Fig. 1 (which is readily removable for standard use).

Our technique has been developed using rat liver perfused with 2% osmic acid in the manner of Claude and Fullam[6] who have published micrographs of guinea pig liver sectioned with the high speed microtome. Small blocks of tissue (approximately one mm cubes) are run up through the alcohol series, into ether-alcohol, then into 3, 6, and finally 10% collodion (Mallinckrodt Parlodion) dissolved in ether-alcohol. The collodion is hardened in chloroform, and the blocks transferred to xylol by way of carbol-xylol. They are then infiltrated with 65°C paraffin. The blocks are finally mounted rigidly in the microtome after being pared down to present a face about one mm square.

To cut 0.2 micron sections it is hardly necessary to say that the knife must be very sharp, but proper stropping is quite adequate. The optimum adjustment of the knife tilt is extremely critical, and even though alignment marks are lined up on the holder, minor adjustments are still necessary every time the knife is moved or replaced.

The sections are cut at moderate speeds, perhaps slightly faster than would ordinarily be used. They tend to rumple somewhat as they are cut. But single sections or short series can be picked up with a fine brush and transferred to a standard specimen screen for the electron microscope. Then, working with a dissecting microscope, one or two corners are tacked down to the screen by pressure with a needle. Gentle teasing with either the brush hairs or with needles will take out the major wrinkles. Finally the section is flattened and given many points of contact with the screen by gently stroking it with the brush, or by rubbing with the blunt polished end of a glass rod. A flattened section on a screen can be seen in Fig. 2.

The final preparation for the electron microscope involves the removal of part or all of the embedding material, with the hazards attendant upon the final evaporation of the solvents. Three methods have been devised, each of which has certain advantages, so that it is worthwhile to consider all.

Method 1. The simplest preparation is to extract the paraffin but leave the collodion in place. This is done by dropping xylol repeatedly on the specimen screen and thus flushing it. It is finally air dried. The collodion which remains is not a serious handicap to observation, at least at a magnification of × 5,000, as it is in the form of a very fine mesh with low density. Since the specimen was impregnated with collodion first, presumably all parts of the section remain supported in place by the collodion during the drying, and in spite of the paraffin extraction.

---

2 von Ardenne, M., *Z. Wiss. Mikroskopie,* 1939, **56**, 8.

3 Sjöstrand, Fritiof, *Nature,* 1943, **151**, 725.

4 O'Brien, H. C., and McKinley, G. M., *Science,* 1943, **98**, 455.

5 Fullam, E. F., and Gessler, A. E., *Rev. Sci. Inst.,* 1946, **17**, 23.

6 Claude, Albert, and Fullam, E. F., *J. Exp. Med.,* 1946, **83**, 499.

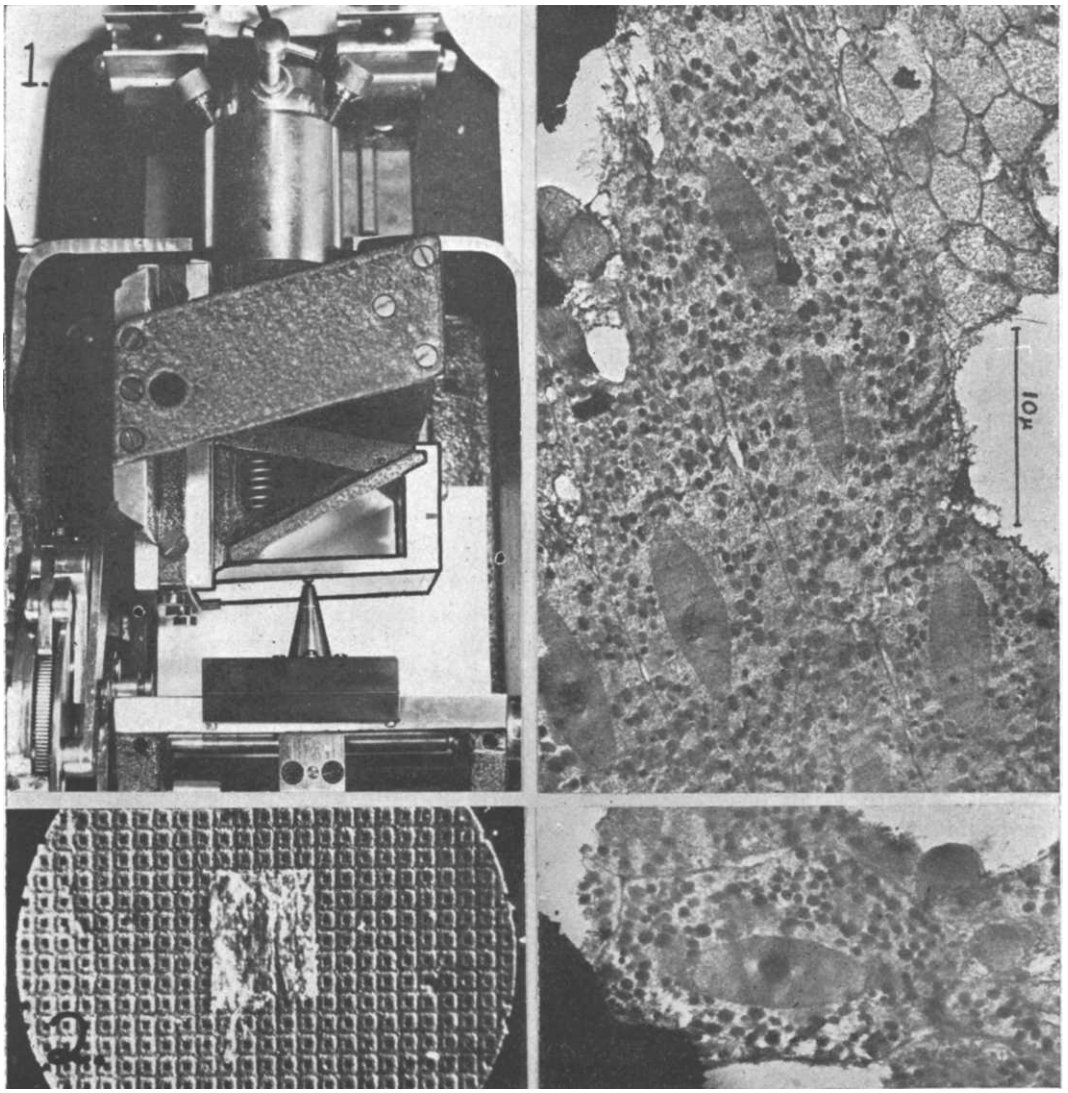
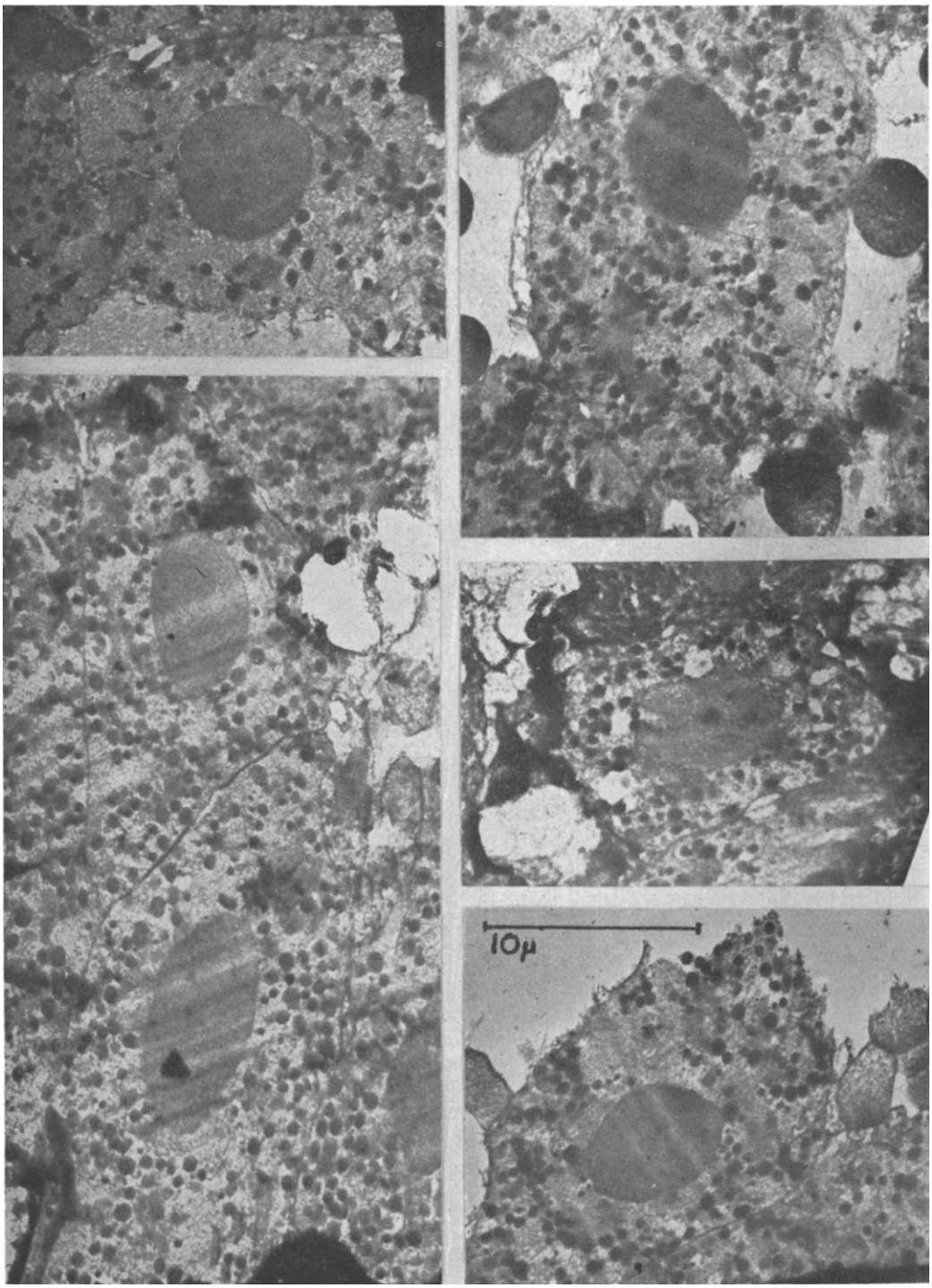


FIG. 1.
Vertical view of Spencer microtome modified by the outlined attachment to cut 0.1 micron.

FIG. 2.
Enlarged view of a 0.2-micron section mounted on a standard 200-mesh specimen screen. The remaining figures show electron micrographs of rat liver sections cut at 0.2 microns. Cell walls, nuclei, nucleoli, mitochondria, bile capillaries, and blood sinusoids are visible. Sections of red blood cells appear in some sinusoids, although most were removed by the perfusion technic employed in the initial fixation with osmic acid.

Such specimens are very strong and resistant to electron bombardment.

Method 2. If the embedding media are to be entirely extracted surface tension may have serious distorting effects during the final drying of the solvents. However, if benzene is used as the final solvent it can be frozen (5°C) and sublimed from the solid state thus minimizing the danger. Adherence of section to screen is none too good during this treatment. Best results are obtained by observing the following points. The screen, with the section tacked on, is inverted on a clean glass slide, and held down with a


10μ

needle. Amyl acetate is dropped on top and the screen flushed, thus removing the collodion. The section is very likely to become detached at this time, and surely will later if the screen is not now squeezed against the section once more. After rubbing the back of the screen with a blunt instrument the section sticks to the screen rather than to the glass, and the screen can be picked up and transferred to benzene which will remove the paraffin. Finally a drop of benzene surrounding the screen is frozen, and the benzene sublimed *in vacuo*. After such a total extraction the section is fragile and does not stand up well under the electron beam. This method is desirable particularly when the fine structure of a supporting medium might introduce uncertainty in the interpretation of the micrographs.

Method 3. In certain situations it presumably would be desirable to prepare serial sections. It is possible to do this, at least with short series, by varying the process as follows. Instead of tacking the sections to screens, they are flattened against a clean glass slide by teasing and brushing. Amyl acetate is flowed over them, followed by benzene, and finally amyl acetate once more. Then the slide is dipped in a 1% solution of collodion in amyl acetate and drained. The sections adhere to the glass throughout this process and are embedded in a thin film of collodion which is then dried. The slide is now dipped in distilled water thus floating the film off on the surface. Screens are then placed over the portions of the film containing sections, and are finally picked up and handled by usual techniques. Drying occurs only after the film is in place for support. A number of sections can be prepared simultaneously in this way. The paraffin is removed entirely, and the original network of collodion is replaced by a thin amorphous film that is none the less quite tough.

The question of artefacts introduced by the method remains to be considered. The knife edge, as it cuts, has little tendency to compress the section. Some distortion may result from drying, but in most parts of a section is scarcely noticeable. The sections are not entirely uniform in thickness, but the variations are estimated to be usually less than .05 micron. Due to the non-uniformity one can find local areas that are probably less than 0.1 micron thick. Lines resulting from the chatter of the knife edge are sometimes present, and easily recognized.

The principal artefacts are certainly those of the original fixation as Claude and Fullam[6] recognized in their work. In spite of very different treatments subsequent to fixation our micrographs are almost identical with theirs. Fixation artefacts become serious at magnifications above $\times$ 5000 when it can be seen that much of the fine structure consists of precipitated fibers. The full potentialities of section cutting for the electron microscope, therefore, will be realized only after the development of superior methods of initial treatment.

Summary. Conventional histological techniques have been modified so that is is possible consistently to cut 0.2 micron sections for use with the electron microscope. The material must be doubly embedded in strong collodion and hard paraffin. The face of the block to be cut must be small, and the tilt of the knife must be precisely adjusted.

Procedures have been developed for partly or wholly removing the embedding media, and mounting the sections for the electron microscope.

Micrographs of rat liver sections show that the principal artefacts are due to fixation rather than subsequent treatments.