SYMPOSIA OF THE
INTERNATIONAL SOCIETY FOR CELL BIOLOGY

1

The Interpretation *of* Ultrastructure

Edited by
R. J. C. HARRIS
Division of Experimental Biology and Virology
Imperial Cancer Research Fund, London

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PREFACE

ULTRASTRUCTURAL investigations by very different methods are now one of the foremost preoccupations of modern cell biology. Visualization of cellular ultrastructures was impossible with the light microscope but became, nevertheless, very urgent because virology, microbiology, developmental biology and cytogenetics required it. In all these fields the ultrastructure of living organisms seemed to comprise the elementary biochemical processes which are directly involved in cellular activity.

During the past ten or twelve years electron microscopy had seemed to satisfy all the requirements of cell biologists for a better understanding of vital phenomena. An ever-increasing number of publications gave innumerable data on ultrastructure. But, at the same time, a stronger need developed for general hypotheses and guiding principles. Critical biologists began to ask What are artifacts? and What are reliable equivalents of living matter? How can we judge electron micrographs: are images which seem simply beautiful to us also true?

This general tendency among such investigators led us to organize a symposium on the interpretation of ultrastructure. We wanted to arrive at a critical evaluation and a full incorporation of many data into general notions. A more biological attitude also seemed to be desirable. The functional and evolutionary aspects of living cells had to be considered in evaluating ultrastructural results. In 1938 Frey-Wyssling linked macromolecular aspects to cellular biology; now the functional and dynamic role of cell constituents cannot be omitted any longer. Manifold techniques are in use today and these had to be considered critically at the symposium, e.g. fixation, control of observations by light microscopy, cytochemistry at the electron microscope level, reversed staining, ultrastructure in embryos, and in plants.

The results of the symposium have strengthened the view that ultrastructural research, critically evaluated, will open new ways to the study of life. We hope that this volume will stimulate international ultrastructural research into cells as units of life.

July 1962 F. E. LEHMANN

CONTENTS

CHEMICAL FIXATIVES FOR ELECTRON MICROSCOPY

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IT is well known that the "standard" osmium tetroxide fixative fails to fix satisfactorily some types of cells, such as the yeast cell and most other plant cells, as well as egg cells of many invertebrate species. Electron microscopists working with these types of cells have resorted to various alternatives of the "standard technique". It was thought valuable to try several of the alternatives on a single type of cell, preferably an easily fixed cell such as that of the rat liver, which is also familiar to most electron microscopists. The fixation techniques to be presented here represent rather drastic deviations from the conventional techniques; they include methods described in electron microscopical literature as well as some variations performed recently by the author.

Osmium Fixatives

The "standard fixative" in Dr. Sjöstrand's laboratory, where this work was begun, can be said to consist of 1 per cent osmium tetroxide dissolved in a medium similar to the physiological extracellular fluid [20]. In the case of the sea urchin egg, the first material to be tried by the author, the physiological vehicle for the osmium tetroxide is sea water. Although 1 per cent osmium tetroxide dissolved in sea water is as good a fixative as any that has been tried on sea urchin eggs, the cytoplasmic framework does not seem to be fixed in a reliable way. Modifications of salt concentration, pH, temperature, etc., had little effect on the final appearance of the cell. A more radical step was to abandon the idea of a vehicle for the osmium tetroxide and to use melted osmium tetroxide by itself as a fixation fluid [1]. At a temperature of 40°C the osmium tetroxide melts and the tissue specimen to be fixed can be floated on a drop of this fixative. This fixation technique has been tried only a few times, since the results did not differ markedly from those obtained after conventional fixation (Fig. 1). An advantage is that the resulting pictures have a higher contrast than is usually obtained; a disadvantage is the deposit of substances, possibly metallic osmium, which are sometimes observed on the outside of some cell membranes.

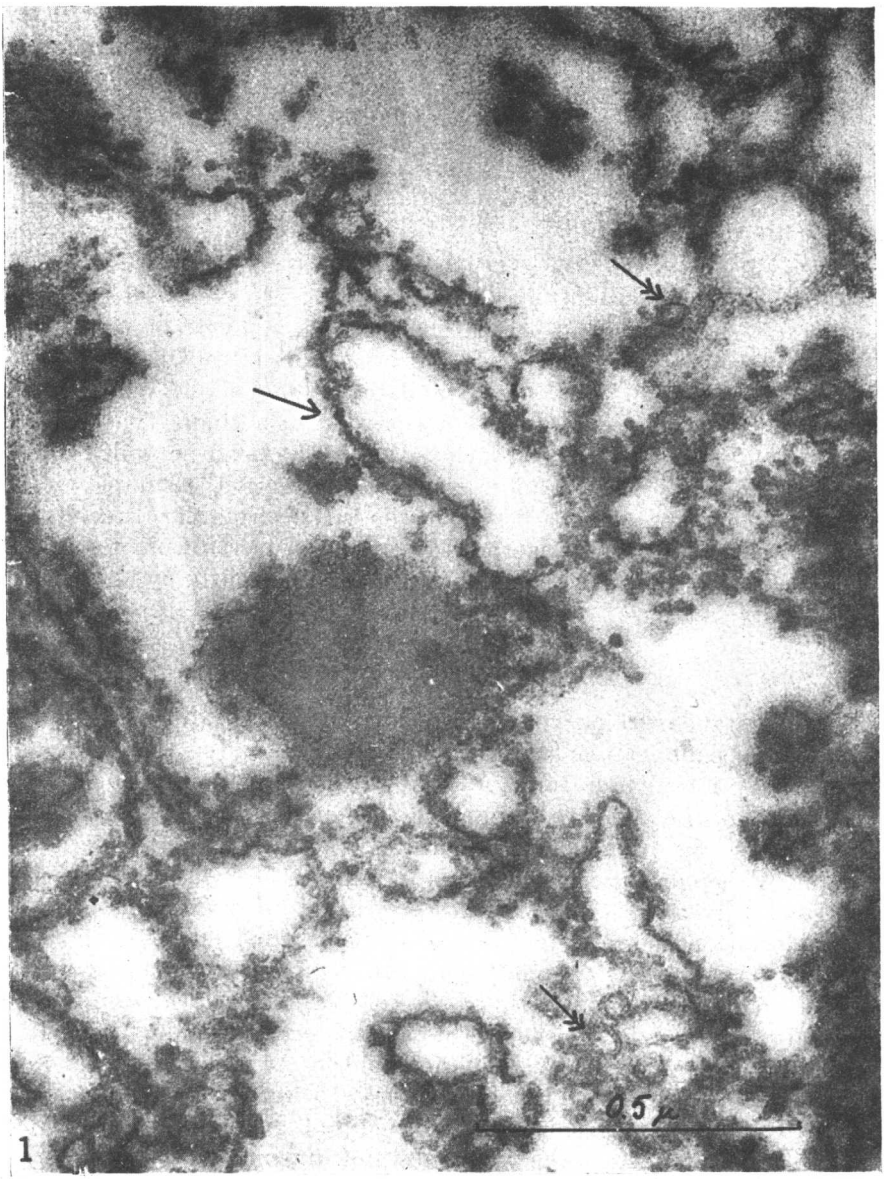

FIG. 1.—Fixation with a drop of pure melted osmium tetroxide. The picture shows a small portion of the cytoplasm from a sea urchin egg. Some of the cytoplasmic membranes appear as double lines (double-barbed arrows), others as single lines (single-barbed arrow). In the centre of the field there is an oil droplet. × 86,000.

In another modification of the fixation technique the osmium tetroxide is dissolved in carbon tetrachloride rather than in an aqueous solvent [2, 3]. A 40 per cent solution has been used. The block of tissue to be processed floats on top of the carbon tetrachloride-osmium mixture. The carbon tetrachloride is prevented from entering the tissue to any large extent by the tissue fluid blanket that still surrounds the tissue block. The tissue blackens rapidly, however, which shows that the tissue fluid has taken up osmium tetroxide. At the concentrations employed the osmium tetroxide has a distribution coefficient between carbon tetrachloride and water of about 100 [5], and the osmium tetroxide concentration in the aqueous solution that permeates the tissue block will be about 0·4 per cent.

The carbon tetrachloride-osmium method, as well as the method using melted osmium tetroxide, fixes the tissue rapidly and results in pictures with a high contrast (Figs. 2, 4, as well as Figs. 1–7 in [3] and Fig. 1 in [4]). The cytoplasmic membranes stand out with unusual clarity and some of them appear definitely double-contoured, whereas other membrane systems, e.g. the ergastoplasmic membranes, appear single at the resolutions achieved in this study. The two categories of membranes also differ in width, 90 Å and 55 Å respectively. These values are greater than those obtained from "standard" osmium pictures. The observations are similar to those of Karrer [11] and seem to speak against the concept of the unit membrane [18] or at least against the idea that all membranes in the cell have unit width and appearance. The carbon tetrachloride-osmium fixation has been successfully applied to sea urchin spermatozoa [3] as well as to *Limulus* and squid spermatozoa [unpublished], sea urchin eggs [2], mussel gills [4] ctenophore tissue (Fig. 2), rat liver (Fig. 4), chicken glycogen body and mitochondrial fractions. In one case (light organ of the firefly) this fixation method resulted in a compact block that was difficult to cut. When comparing liver fixed in carbon tetrachloride-osmium with that fixed in the standard fixative, the differences are not very striking (Fig. 3).

Other solvents have been tried as vehicles for the osmium tetroxide: acetone, pyridine and glycerol. These fluids are miscible with water and the fixatives have quite different properties from those of the osmium tetroxide-carbon tetrachloride mixture. Osmium tetroxide is only slightly soluble in glycerol (*c.* 3 per cent) and micrographs obtained with this fixative have a very low contrast. Sections obtained from blocks fixed in glycerol solutions of osmium tetroxide appear similar to sections cut from blocks fixed by standard methods, although the lack of contrast prevents detailed comparisons. The mitochondrial cristae

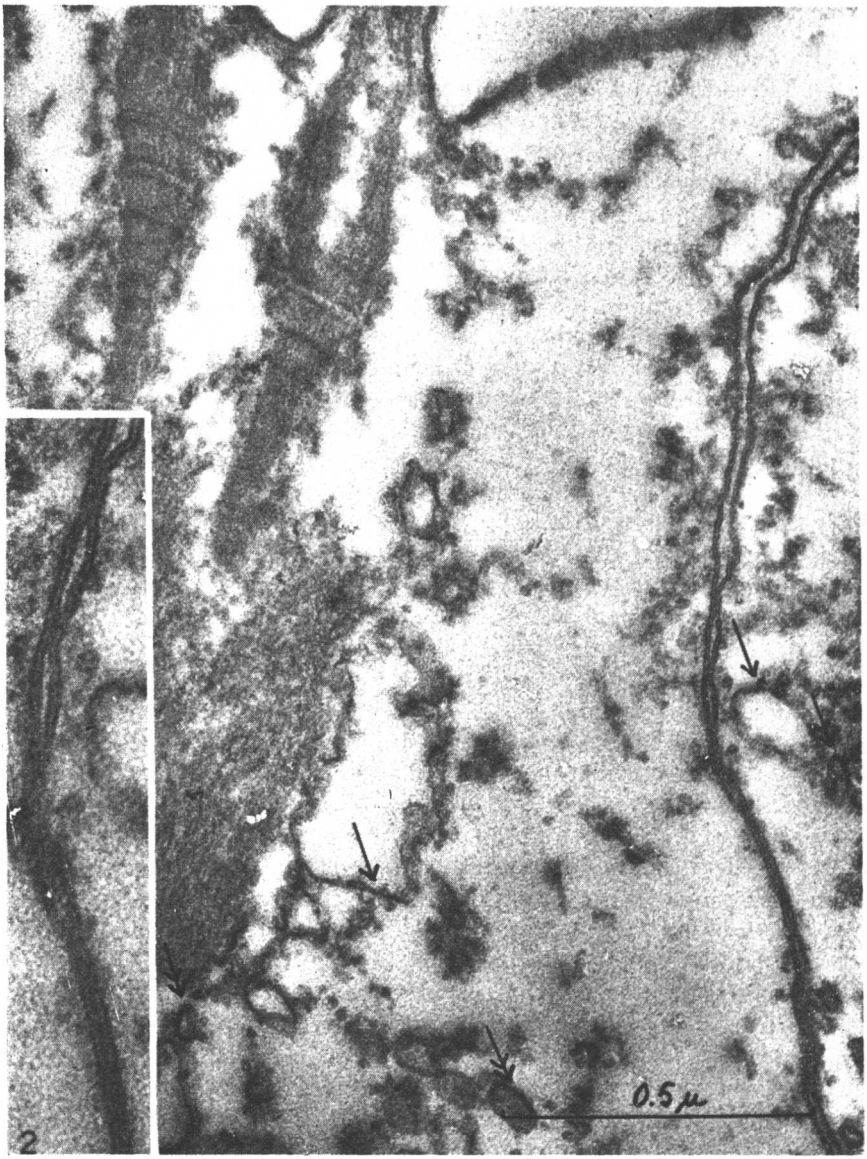

FIG. 2.—Fixation with 40 per cent osmium tetroxide in carbon tetrachloride. The figure shows the apical region of a ciliated cell from the ctenophore *Mnemiopsis leidyi*, with the ciliary basal bodies and rootlets at the upper left and a cell border along the right margin. Double-contoured cytoplasmic membranes are marked with double-barbed arrows, single membranes with single-barbed arrows. The inset shows, at a higher magnification, the intimate association of the two adjacent cell membranes. × 83,000; inset × 170,000.

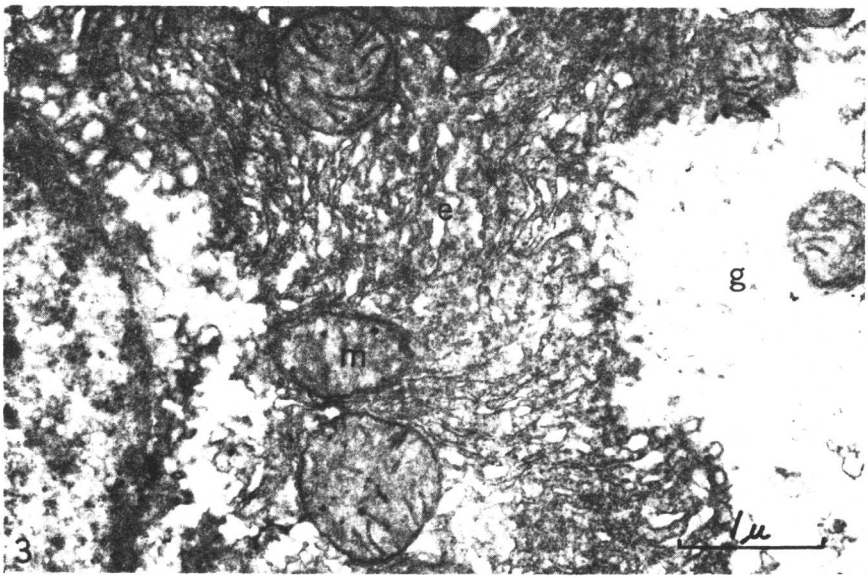

FIG. 3.—Rat liver cell cytoplasm after fixation in 1 per cent osmium tetroxide in buffered Tyrode solution. A portion of the nucleus is to the left, ergastoplasmic membranes at e, glycogen areas at g, and a mitochondrion at m. × 20,000.

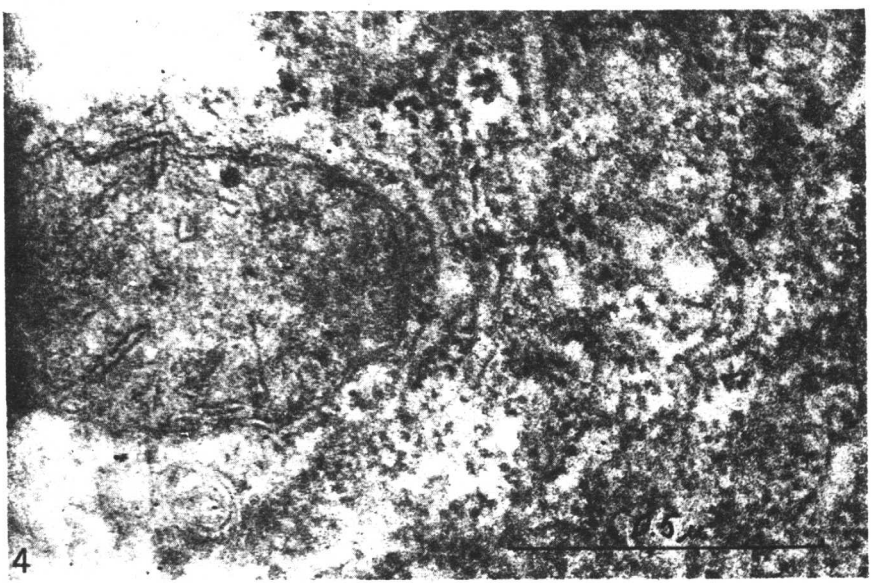

FIG. 4.—Rat liver cytoplasm after fixation in 40 per cent osmium tetroxide in carbon tetrachloride. A mitochondrion is seen at the left and ergastoplasmic membranes to the right. × 84,000.

may appear in reversed contrast. With pyridine or acetone used as solvents (in fixations at 0°C) the results have been more discouraging, since the sectioned cells were barely recognizable.

Some osmium fixatives described in the literature will be mentioned here. In freeze-substitution techniques the freshly frozen tissue is transferred to a substituting fluid that is commonly kept at — 40°C or at — 70°C. This fluid replaces the water of the tissue. It has been demonstrated by Feder and Sidman [8] that the use of chemical fixatives in the substitution fluid improves the quality of tissue preservation. They found that 1 per cent osmium tetroxide dissolved in acetone was an excellent substitution fluid. This technique has been adapted for electron microscopy by Fernández-Morán [9] and by Rebhun [17], to whose papers the reader is referred.

Anhydrous osmium tetroxide vapour has been used in connection with either freeze-drying or air-drying techniques. In the hands of Hanzon and Hermodsson [10] the osmium vapour-stabilized, frozen-dried tissue has an appearance quite similar to that obtained after a conventional osmium fixation, with the exception that the ribosomal particles are not visible.

Schidlovsky [19] working on air-dried chloroplasts shows that an osmium tetroxide vapour fixation, introduced either before or after the air-drying, results in micrographs that show a negative or reversed contrast.

Permanganate Fixatives

An aqueous solution of potassium permanganate was first introduced as a fixative for electron microscopy by Luft [12] in 1956. For various reasons this fixative has come to be the most commonly employed alternative to osmium fixation. Despite its widespread use, there has not yet been published a detailed comparison of the results obtained by using the two fixatives. It is not intended to catalogue the similarities and differences, but mention will be made of some of the more striking differences noted for rat liver.

After potassium permanganate fixation the cell borders do not take the same tortuous course as after osmium fixation. The ergastoplasmic membranes (α-cytomembranes, granular endoplasmic reticulum) are relatively straight, and no membranes closely follow the contours of mitochondria or the cell membrane. There is a separate category of cytoplasmic membrane that has no clear equivalent in the osmium pictures; this is a straight dense membrane that crosses the fields of ergastoplasmic membranes or the glycogen areas. The Golgi apparatus

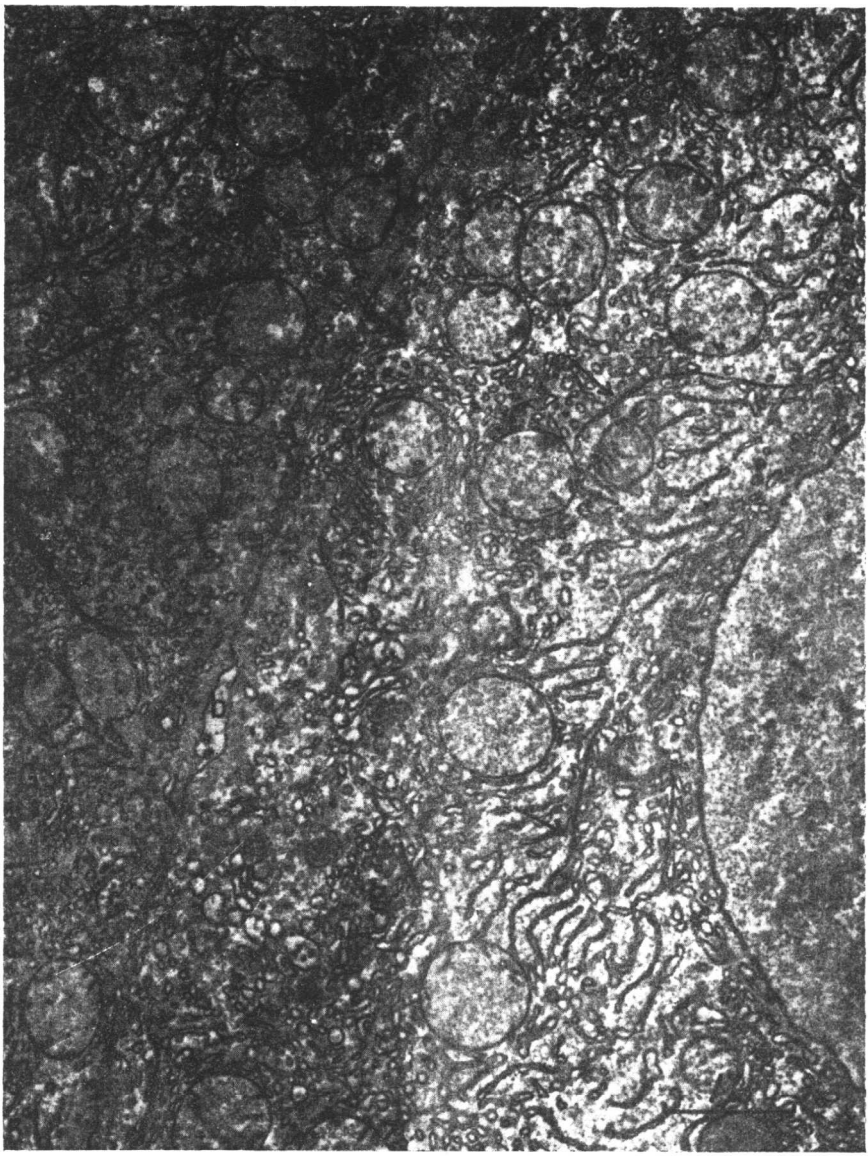

FIG. 5.—Rat liver cytoplasm after fixation in 1 per cent potassium permanganate in buffered Tyrode solution. The ergastoplasmic membranes (e) have the same density as the vesicles in the glycogen areas (g). There is also another category of membrane, the straight dense lines indicated by arrows. At G is the Golgi apparatus, at b a bile duct. × 17,000.

contains small vesicles rather than granules; the mitochondria are swollen and the average number of cross-sectioned inner membranes (cristae) is reduced from about 20 in osmium-fixed liver to between 4 and 8; the glycogen areas contain many more vesicles than have been seen after osmium fixation; there is a narrow and moderately electron-dense ectoplasmic zone near the cell membrane; the lipid granules are totally extracted or nearly so; the nucleus has a washed-out appearance; the cytoplasmic and nuclear background contains an abundance of small dark granules; the ribosomes are not visible; all membranes seem to have a uniform width and are relatively easily resolved to show a double-contoured appearance (Fig. 5).

These differences are listed, not to show that potassium permanganate fixation is inferior or superior to osmium fixation, but only to demonstrate that the two fluids have widely different fixation qualities.

Luft concluded that the fixing agent is the permanganate ion, and that the species of cation is of relatively little or no importance. It will be shown that this is not quite correct.

Mention will first be made of the suggestion by Wetzel [23] that the sodium permanganate solution, because of its greater similarity to a "physiological" extracellular fluid, might be a better fixative than the potassium salt. In his experience, sodium permanganate fixation consistently gave better preservation, especially with regard to the cell membrane.

Dr. H. H. Mollenhauer has stated that different permanganates have different fixation properties [15]. In order to compare the effects of these reagents I have fixed liver tissue and sea urchin eggs in a saturated (3 per cent) potassium permanganate solution as well as in 4 per cent solutions of calcium, barium and zinc permanganate in distilled water. From a chemical point of view the permanganates differ in stability and solubility; the sodium permanganate solutions decompose more rapidly than those of potassium permanganate. From a theoretical point of view the use of calcium permanganate would seem to be quite promising since, according to Baker [6], the calcium ion has a stabilizing action on lipids.

The results will be mentioned briefly although the data are still preliminary. After calcium permanganate fixation all the membranes

FIG. 6.—Rat liver cytoplasm after fixation in a 4 per cent solution of calcium permanganate in distilled water. All membrane systems in the cell appear as clearly double-contoured and uniformly thick lamellae. The width of these double membranes is 100 Å. The inner and outer membranes of the mitochondria, however, appear as triple lines, the middle line of which is thicker than the others. The particle to the right is a microbody (a cytosome), which after this fixation characteristically contains a striated inclusion body. × 77,000.

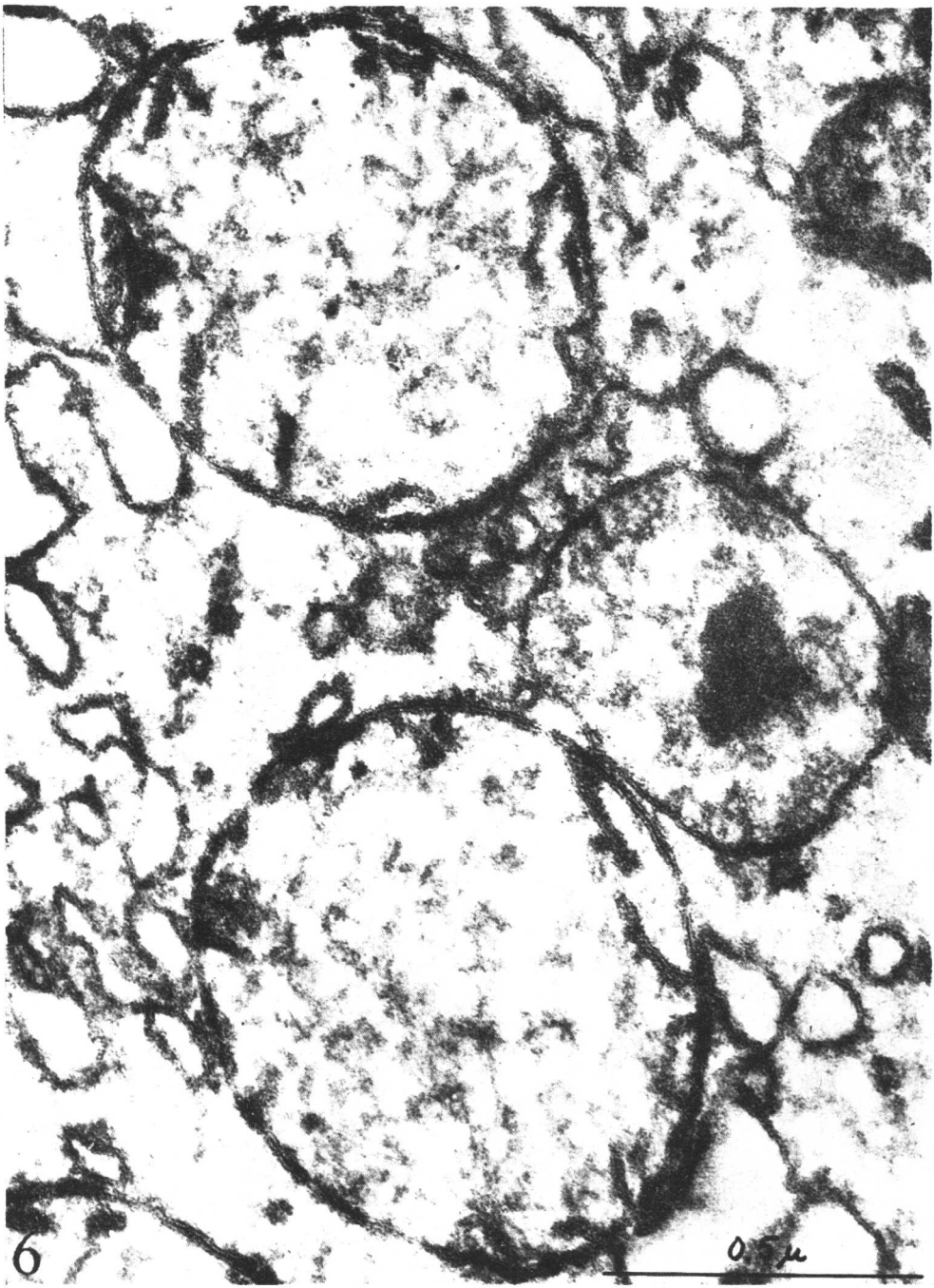

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FIG. 6.