A STUDY OF FIXATION FOR ELECTRON MICROSCOPY

By G. E. PALADE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 19 TO 23

(Received for publication, November 5, 1951)

Microtomy for electron microscope studies has recently shown considerable progress due to a number of improvements such as the reduction of the advancing rate of the usual microtomes (1, 2), the embedding in hard plastics (3), and the introduction of glass knives (4). As a result of this progress, it is now possible to obtain sections of suitable thinness in sufficient yield to permit systematic study of practically any type of cell *in situ*. An appreciable number of electron microscope studies on sectioned material have already been published. Although they have provided interesting morphological information, the results with the technique have not thus far come up to expectations, particularly in the range of fine structure. This is due, in general, to the poor fixation of the specimens, although, in most instances, fixatives known to give good results in light microscopy have been used. It was soon realized, even before sectioning became practical, that as far as fixation is concerned, electron microscopy is going to be more exacting than light microscopy.

Using tissue cultured cells, Porter, Claude, and Fullam (5), Porter (6), and later Barer (7), found that the cell morphology, as revealed by the electron microscope, is influenced to a considerable extent by the fixative employed. The authors first mentioned (5, 6) concluded that the best fixation was obtainable by treatment with OsO_4 vapors. This was stressed again recently by Porter and Kallman (8), who, in addition, studied in detail the influence of the duration of OsO_4 fixation upon the morphology of the specimen. In some of the studies on sectioned material, OsO_4 solutions were used as a fixative, but the results appeared to be less satisfactory in tissue blocks than in tissue cultures, even when the fixation was performed by perfusion. In the opinion of the writer, the addition of other reagents, such as lanthanum sulfate and potassium dichromate, to the OsO_4 solutions (9), does not seem to improve the situation materially.

The present paper reports a series of experiments conducted in order to find better conditions for the fixation of small blocks of tissue in OsO_4 solutions. The experiments indicate that: (*a*) in tissue blocks, OsO_4 fixation is preceded by an acidification of the tissue, (*b*) the quality of OsO_4 fixation is highly dependent on the pH, and (*c*) the fixation can be considerably improved by buffering the OsO_4 solutions at pH 7.3–7.5 with acetate-veronal buffer.

Materials and Methods

In all experiments, organs of adult, new born, and embryonic albino rats (Wistar strain) were used.

Specimen Collection.—The animals were anesthetized with ether, the organ supplying the tissue was operatively exposed, and a piece of it excised with curved scissors. This piece was put on a strip of cardboard, covered immediately with a drop of fixative, and, while immersed in it, trimmed down with a sharp knife to blocks of 1 c.mm. or less. By operating thus, only a few seconds passed from the severance of the blood connections of a specimen until the beginning of its fixation.

Fixation.—3 to 4 such small tissue blocks were transferred on a strip of filter paper into a vessel provided with a ground glass stopper and containing 3 to 4 ml. of fixative solution. For fixation the following solutions were used:—

(*a*) 1 or 2 per cent OsO_4 in double distilled water; the pH varied from 5.6–6.3.

(*b*) 1 or 2 per cent OsO_4 buffered at various pH's from 3.0–9.0 with acetate-veronal buffer (10). In the majority of cases, the final concentration of Na acetate and Na veronal was kept at 0.028 M (0.14 M Na acetate and Na veronal, 5 ml.; 0.1 N HCl, α ml.[1]; 2 per cent OsO_4, 12.5 ml.; make up to 25 ml. with distilled water). For low pH's, or for a final 2 per cent OsO_4 concentration, a 4 per cent OsO_4 stock solution was used. In a few cases, the final concentration of Na acetate and Na veronal was raised to 0.047 M. This was made possible by using, for the preparation of the buffer, 0.2 N HCl instead of the usual 0.1 N HCl.

(*c*) 1 per cent OsO_4 buffered at various pH's from 6.0–8.0 with KH_2PO_4–K_2HPO_4; final buffer concentrations: 0.05 M and 0.028 M. The pH's were controlled with a Beckman glass electrode pH meter.

All specimens were fixed for 4 hours at room temperature. Thereafter the blocks were washed for approximately 1 hour in 2 to 3 changes of distilled water and transferred to 70 per cent ethanol.

Dehydration.—For dehydration, 70 per cent, 95 per cent, and 100 per cent ethanol were used, the tissue blocks being kept 1 hour in each. A longer, even an overnight stay in 70 or 95 per cent ethanol, did not appear to effect any changes.

Embedding.—From the absolute ethanol the tissue blocks were transferred to a mixture containing equal volumes of *n*-butyl methacrylate[2] and ethanol and kept therein for 1 hour. They were soaked thereafter for 3 hours in 3 changes of pure monomeric *n*-butyl methacrylate and finally embedded as recommended by Newman *et al.* (3) using butyl methacrylate as a plastic, and 2,4-dichlorobenzoyl peroxide[3] as a catalyser at 47°C. A stay of 24 hours at this temperature was in general sufficient to bring about a suitable polymerization. If the plastic blocks were too soft for cutting at once, they were kept for an additional 24 to 48 hour period at 47°C. Keeping the plastic blocks at room temperature for a few days before sectioning, rendered them easier to cut. It is assumed that in both cases a more complete polymerization occurred.

Sectioning.—The microtome used was built by Claude and Blum (11) in the Rockefeller Institute and adapted recently to the use of glass knives. The machine provided a continuous advancing movement of the specimen, which revolved in front of a stationary knife. In order to insure a smooth advance of the block, the microtome was operated by a motor (12). The sections from the knife were floated on a liquid surface in order to facilitate spreading. The

[1] Amount varying with the pH; for pH 7.4 α = 5.0 ml.

[2] Obtainable either from Rohm and Hass Co., Philadelphia, or from du Pont de Nemours & Co., Polychemicals Department, New York City.

[3] Obtainable from Novadel-Agene Corp., Lucidol Division, Buffalo. It is sold in paste form mixed with 50 per cent dibutyl phthalate (a plasticizer) under the trade name luperco CDB.

liquid, namely 50 per cent acetone in water, was kept in a paraffin trough built behind the cutting edge of the knife. For this study, the microtome was set to cut either at 0.1 μ or at 0.06 μ. Nevertheless there was considerable variation in the thickness of the sections obtained, as demonstrated by differences in their interference colors and their transparency under the electron beam.

Mounting.—From the liquid surface, the sections were lifted directly on to electron microscope screens (150 copper electro mesh), placed on filter paper, and allowed to dry. The screens were previously coated with a film made from a 0.075 per cent formvar solution. This procedure saved a considerable number of preparations from destruction or deformation during examination in the electron microscope, for sections mounted on uncoated screens very often melted or shrivelled under the electron beam. As is known, the *n*-butyl methacrylate is a thermoplastic resin with a softening point around 33°C. (13).

The mounted preparations were screened under the light microscope and the selected ones stored in a desiccator over phosphorus pentoxide until examination. No attempt was made to remove the embedding plastic from the sections after mounting. In agreement with Hillier and Gettner (12), it was felt that such a procedure could expose the preparations to considerable surface tension forces and thereby produce drying artifacts of the type discussed by Anderson (14).

Microscopy.—The sectioned material was examined in a RCA (model EMU) electron microscope with a 30 μ aperture in the objective lens. The electron micrographs were taken at an original magnification of 3,000 × or higher and enlarged thereafter photographically, as desired.

Other Techniques.—1. Tissue homogenization: The effects of OsO_4 fixation were also studied on liver homogenates. The latter were prepared by homogenizing fresh liver pulp directly in cold (0°–3°C.), buffered or unbuffered, 1 per cent OsO_4 solutions by means of an all glass apparatus (15). The ratio of liver pulp to fixative solution was 1:10 or 1:20. With a short homogenization (30 to 45 seconds), most of the liver cells remained intact in clumps of various sizes. The morphological changes produced by fixation were studied with the light or phase-contrast microscope. The pH of each OsO_4 solution and of the respective homogenate were taken with a Beckman pH meter at room temperature after suitable equilibration.

2. Slice preparations (16) were used in order to learn whether acidification of the tissue precedes its fixation by OsO_4. For this purpose, 4 to 5 ml. of a 1 per cent neutral red solution was injected intraperitoneally into an albino rat. 2 or 3 hours later, when the skin was deeply colored, the animal was anesthetized with ether, the liver exposed, and free hand sections of it mounted under compression between slide and coverslip. The latter was secured only at the corners with vaseline drops and the preparations were immediately immersed in a 1 per cent OsO_4 solution. Such fresh liver sections appeared colored in orange-red (salmon red) to the naked eye. Under the microscope, the liver cells showed in some cases a considerable number of vacuoles stained orange-red or red, in an otherwise unstained cytoplasm; in other cases the cytoplasm appeared diffusely stained orange with only a few droplets or vacuoles stained the same color. After immersion into the fixative, the latter penetrated between slide and coverslip and began to diffuse slowly (less than 1 mm. per hour) into the liver slice. The diffusion process could be easily followed because the regions of the slice already penetrated by OsO_4 became brown or black due to the formation (by interaction with the tissue) of lower, deeply colored osmium oxides. In advance of the diffusion wave of OsO_4, the tissue turned to pink from the original orange-red color. This change, the significance of which will be discussed later, was particularly noticeable under the microscope in cases showing originally a diffuse orange staining of the cytoplasm. The pink color did not appear to be due to an interaction of OsO_4 with the dye because: (*a*) OsO_4 did not affect the color of neutral red *in vitro*, and (*b*) in liver sections, osmium oxides rendered the neutral red stained cytoplasm light brown and its colored vacuoles dark brown or black.

It is felt that slice preparations like those described should provide information about diffusion processes and pH changes occurring in a tissue block during fixation.

OBSERVATIONS AND EXPERIMENTS

Fig. 5 shows the electron micrograph of a section through a hepatic parenchymal cell. The section was cut from a block of liver fixed in 1 per cent OsO_4 and processed thereafter as already described. The nucleus of the cell shows a certain amount of reticular precipitation. The mitochondria, appearing mostly in cross-section, seem to be swollen; the mean diameter for 100 measurements is 0.70 μ. The lipid inclusions are difficult to differentiate from the mitochondria. The ground substance is precipitated in a network partially resolved by the electron microscope and shows in numerous places large empty vacuoles. The latter are more frequent within or around some bundles of elongated structures that appear to be embedded in the ground substance. The electron micrograph does not show clearly whether these structures are filamentous or canalicular in character. At the time of fixation, this cell was situated at the periphery of the block. In cells more centrally located, the swelling of the mitochondria and the precipitation and vacuolization of the ground substance of the cytoplasm were more pronounced. In the center of some blocks, a coarse, reticular precipitate of the nuclear content was also visible.

Previous experience with tissue homogenates has shown that some of these morphological alterations, namely those coming within the resolution limit of the light microscope, *e.g.* mitochondrial swelling and nuclear precipitation, can be experimentally induced by acidification at definite pH's. For instance, the nuclear content precipitates regularly around pH 5.8. This suggests that in tissue blocks fixation by osmium oxides is preceded by an acidification of the tissue, and the fact that the penetration of OsO_4 is very slow (17) accords with this suggestion. One might think that, by reducing the depth OsO_4 has to penetrate into the tissue, the quality of fixation could be improved, but attempts made in this direction were not successful. After short homogenization of liver pulp in 1 per cent OsO_4 solutions, similar morphological changes were found, although, in this instance, OsO_4 had to penetrate only into isolated cells or into clumps consisting of a few cells. In fact, the changes appeared to be more pronounced in liver homogenates than in liver blocks, and as such they could be easily detected in the light or phase-contrast microscope. The pH of the liver homogenates was found to be consistently lower than that of the original OsO_4 solutions. 4 hours after homogenization, the difference was a whole pH unit or more, and it increased to approximately 1.5 units in 24 hours. Assuming that the original pH of the liver was close to neutrality, it may be inferred that a considerable acidification of the tissue occurred during homogenization in OsO_4 solutions. It must be admitted however, that this change in reaction may not necessarily be connected with OsO_4 fixation, but might be due to the production

of an acid of injury, a common occurrence with tissues (18) and cells (19) after various mechanical insults including pulping and homogenization (20).

In order to find out if an acidification of the tissue is actually associated with OsO_4 fixation another approach was tried, namely that provided by slice preparations obtained as indicated in a preceding section. In their case, the amount of mechanical damage due to cutting and mounting was less than on tissue homogenization, and roughly comparable to that undergone by ordinary tissue blocks. In addition, the neutral red present inside the cells, constituted a good indicator of pH changes. Before immersion in 1 per cent OsO_4, the red-orange (salmon red) color of the liver slices showed that their pH was probably $\geqslant 6.8$. Soon after immersion, as already stated, a dark brown rim, due to the diffusion and reduction of OsO_4 into the tissue, appeared at the periphery of the slices. Inside the brown rim, a pink ring differentiated progressively, indicating that the pH of the tissue was dropping in front of the diffusion wave of osmium oxides. Under the microscope, the liver cells within this pink ring showed regularly a diffuse pink color of the cytoplasm, regardless of the diffuse, or vacuolar type of staining showed by the cells in the center of the slice. According to Parat (21), the diffuse pink coloring corresponds to a pH of $\leqslant 6.0$. The pink ring began to be noticeable under the microscope 10 to 15 minutes after immersion and became broad enough to be seen with the naked eye after 30 to 45 minutes. The center of the preparation remained orange for 45 to 60 minutes and turned thereafter progressively yellow (probable pH $\geqslant 8.0$).

It is difficult to ascertain whether within liver cells the various neutral red colors indicate the same pH's as *in vitro*, under controlled conditions. Available data (22, 23) obtained with phthalein indicators and concerning the H^+ activity of liver cell cytoplasm, are rather conflicting. Accordingly, the experiments with slice preparations did not permit any definite conclusion as to the exact value of the pH of the liver cell cytoplasm. They indicated, however, that a difference in pH was established among the various zones of a tissue slice during immersion in OsO_4. The center was probably neutral or alkaline, while the periphery became more acid just prior to penetration and fixation by the osmium oxides. It appears, therefore, that in slice preparations, an acidification of the tissue actually precedes fixation by OsO_4. The same may be assumed to obtain for tissue blocks. This finding supports the view that the morphological changes already described in an OsO_4 fixed cell are due to a local increase in H^+ concentration that occurs before fixation. From the experiments with tissue homogenates, it may be inferred that the acidification and its damaging effects upon cell structures cannot be prevented by reducing the dimensions of the tissue blocks below 1 mm. It is clear that, under such conditions, means other than mincing or homogenizing will have to be used in order to prevent the acidification of the tissue.

Influence of Enzyme Inhibitors upon Fixation.—It seemed possible that the

acidification above mentioned may be due to acids produced in the cells by some enzymes, the activity of which is enhanced by the severance of blood connections. This hypothesis was tested by adding to the OsO_4 solutions substances (0.04 M iodoacetamide and 0.04 M potassium fluoride) known to inhibit acid-producing metabolic processes; *e.g.*, anaerobic glycolysis and hydrolysis of phosphoric acid esters. Mixtures of OsO_4 and such enzyme inhibitors were used both for preparing liver homogenates and for fixing liver blocks. The results were compared with those obtained in similar preparations with plain OsO_4. It was found that the addition of the enzyme inhibitors mentioned did not prevent the acidification of liver homogenates and did not result in a better preservation of cell structures as revealed in sections by the electron microscope.

This failure prompted another series of experiments in which the effects of various pH's and buffers upon OsO_4 fixation were studied. The experiments were conducted in such a way as to determine: (*a*) if the pH of the OsO_4 solution has any influence upon the appearance of the fixed cells and (*b*) if the quality of fixation could be improved by buffering the OsO_4 solutions at a certain pH.

Influence of pH upon OsO_4 Fixation.—A series of small liver blocks obtained under comparable conditions from the same animal were fixed in 1 per cent OsO_4 solutions buffered respectively at pH 5.0, 6.0, 7.0, and 8.0 (± 0.1) with acetate-veronal buffer. The concentration of Na acetate and Na veronal was the same at each pH (0.028 M). The findings in such an experiment are shown in Figs. 1 to 4. They indicated that the appearance of the fixed cells was influenced by the pH of the OsO_4 solution. *The nuclear content* was precipitated in a coarse network at pH 5.0, showed less precipitation at pH 6.0, and appeared more or less homogeneous at pH 7.0 and 8.0. *The mitochondria* seemed to be swollen on both sides of neutrality, the smallest diameter being found at pH 7.0. The mean diameters for 100 measurements were: pH 5.0:0.73 μ; pH 6.0: 0.80 μ; pH 7.0:0.54 μ; pH 8.0:0.60 μ. *The lipid inclusions* retained in general their spherical form at acid and neutral pH's. At pH 8.0, the large lipid inclusions appeared swollen and vacuolated. Many of the small lipid inclusions showed one or more small vacuoles even at acid or neutral pH's. *The ground substance* was precipitated in a coarse network at pH 5.0; at pH 6.0 it showed a much finer precipitation but a considerable number of vacuoles; at pH 7.0 it appeared finely granular, almost homogeneous, with very few or no vacuoles; at pH 8.0 the vacuoles reappeared, while the background remained granular although coarser. The ground substance seemed to scatter more electrons when fixed at neutrality or on the alkaline side than when fixed at acid pH's. This may be due to the fact that at neutral or alkaline pH's it retained more osmium oxides. The effect could also be explained by differences in the state of dispersion of the constituents of the ground substance. Parallel variations in scattering power were shown by the fixed blood plasma found in the lumen of liver

vessels. In the cells fixed at pH 7.0, the elongated structures already mentioned as present in the ground substance showed a definite pipe-like (canalicular) character which was more difficult to recognize at acid pH's. *The cell membranes*, as well as the nuclear and mitochondrial membranes, appeared thicker and denser at acid pH's than at neutral or alkaline reactions.

In other experiments, a broader pH range was studied using the same buffer system, and the same concentration of Na acetate and Na veronal. When the OsO_4 solution was buffered at pH 3.0 and 4.0, the precipitation effects were more pronounced than at pH 5.0; in addition, swelling and vacuolization of the lipid inclusions, and clumping of the mitochondria occurred. At pH 9.0, the fixation was similar to that obtained at pH 8.0, only the vacuolization of the ground substance and of the lipid inclusions appeared to be more frequent. Similar experiments were conducted with OsO_4 solutions buffered at pH 6.0, 7.0, and 8.0 with 0.028 M phosphate buffer. The fixation obtained was comparable to that produced at the same pH's by acetate-veronal buffered OsO_4, but a greater amount of vacuolization of the ground substance and of the lipid inclusions was found at both pH 7.0 and 8.0.

In liver blocks fixed in OsO_4 buffered with either acetate-veronal or phosphate buffer at pH 7.0 or higher, the center of the block consistently showed a more "acid" type of fixation than its periphery. This suggests that during fixation an acid gradient was established towards the center, a fact to be expected in view of known differences in the diffusion rates of the various components of the buffered fixative. In addition, at the periphery of the block a certain amount of disintegration of the tissue was regularly found. The cells had separated as if the intercellular cement had been dissolved or loosened. The most peripheral cells, especially those injured during the trimming of the block, showed disintegrated mitochondria and ground substance canaliculi dispersed in the embedding plastic, facts suggesting that a certain amount of extraction of the tissue block had been brought about by the monomeric *n*-butyl methacrylate before polymerization. In all blocks, however, there was a wide enough "intermediary zone" in which the appearance of the fixed cells was characteristic for the prevailing pH.

These experiments show that the appearance of cells fixed in tissue blocks by OsO_4 is highly dependent on the H^+ activity of the fixing solution. Under such circumstances one could easily speak of an acid, a neutral, and an alkaline type of OsO_4 fixation.

Effects of Buffered versus Unbuffered OsO_4.—In the preceding experiments, the quality of fixation obtained by using 1 per cent OsO_4 buffered at pH 7.0 was the one that came the closest to the quality of OsO_4 fixation obtained in tissue culture material. For reasons to be explained later, the latter can be considered the best cytological fixation obtainable in electron microscopy under present conditions. It was therefore assumed that OsO_4 solutions buffered around neu-

trality might be better fixatives than the usual, unbuffered solutions of this tetroxide. The assumption was tested both on liver homogenates and on liver blocks.

In a series of experiments with homogenates, aliquots of liver pulp obtained from a single animal were homogenized in parallel in unbuffered and buffered OsO_4 solutions. For the latter, the slightly alkaline pH 7.3–7.5 was chosen because in this range the results appeared to be better, *i.e.* closer to the tissue culture standard, than at pH 7.0. In addition to the acetate-veronal and phosphate buffers, the citrate-phosphate buffer (24) was used (3 volumes buffer: 10). The homogenates, prepared as previously explained, were compared under the light or the phase-contrast microscope. The buffered and unbuffered OsO_4 solutions yielded consistent and definite differences in the state of cell preservation produced. Cells fixed in OsO_4 buffered at pH 7.3–7.5 with acetate-veronal or phosphate buffer and examined in the light microscope retained most of the appearance they had in the living state. The citrate-phosphate buffered OsO_4 seemed to be less efficient in this respect, the nuclei appearing swollen and partially precipitated. As already stated, the unbuffered homogenates regularly showed coarse precipitation of the nuclear content and vacuolization of the mitochondria. Frequently, the buffering capacity of the phosphate and acetate-veronal buffers was partially overcome, but the pH of the homogenates never dropped below pH 6.5.

In another series of experiments, small liver blocks obtained from a single animal were fixed in parallel in (*a*) 1 per cent OsO_4 in double distilled water (the pH of this solution was found to vary from 5.6–6.1) and (*b*) 1 per cent OsO_4 buffered at pH 7.3–7.5 with 0.028 M acetate veronal buffer. The results obtained with buffered OsO_4 solutions proved to be consistently better. Figs. 5 and 6 illustrate the findings in such an experiment. In the cells fixed in buffered OsO_4 (Fig. 6) the mitochondria did not appear to be swollen (mean diameter for 100 measurements: 0.55 μ) and could be easily differentiated from the lipid inclusions; the ground substance was homogeneous and rarely showed vacuoles; the elongated structures embedded in it appeared as well preserved canaliculi; the nuclear content had a homogeneous appearance and the nucleoli appeared to have a convoluted structure. The appearance of the cells fixed in unbuffered OsO_4 has already been described (page 288). Swelling of the mitochondria (mean diameter for 100 measurements: 0.70 μ), precipitation and vacuolization of the ground substance, and disruption of its canaliculi were common features for such cells, which, in addition, appeared to be more voluminous than those fixed in buffered OsO_4. This increase in volume might be an effect of hypotonicity (1 per cent OsO_4 = 0.039 M OsO_4). Other experiments with liver blocks showed that the quality of fixation did not seem to improve over that illustrated by Fig. 6 when: (*a*) 2 per cent OsO_4 buffered with acetate-veronal was used instead of 1 per cent; (*b*) the concentration of Na acetate and Na veronal was raised

from 0.028 M to 0.047 M; (*c*) inhibitors such as iodoacetamide and KF were added to the buffered OsO_4; (*d*) the acetate-veronal buffer was replaced by 0.028 M phosphate buffer.

It appears therefore that, in liver blocks, the quality of fixation obtained by using 1 per cent OsO_4 buffered at pH 7.3–7.5 with 0.028 M Na acetate—Na veronal is better than that produced by plain, unbuffered 1 per cent OsO_4. The latter strongly resembles the quality of fixation produced by OsO_4 solutions buffered between pH 5.0 and 6.0. As already indicated, the quality of fixation obtained with buffered OsO_4 solutions is not homogeneous throughout a given block. Well fixed cells are to be found in the "intermediary zone," the best fixed ones immediately below the disrupted periphery of the block.

Additional experiments proved that buffered OsO_4 is better than unbuffered OsO_4 for the fixation of other organs besides liver. Figs. 7 and 8 show the obvious differences obtained with the two fixatives, in the case of the pancreatic exocrine cells. The cells fixed in buffered OsO_4 (Fig. 8) had an impressive number of canaliculi embedded in the ground substance of the cytoplasm and arranged in a complicated pattern. The zymogen granules and the mitochondria did not appear to be swollen. In the cells fixed in unbuffered OsO_4 (Fig. 7) there was marked vacuolization of the ground substance, resulting in the disruption of the pattern of the canalicular system. Swelling of the lipid inclusions was also apparent, corresponding most probably to their transformation into myelin figures (25). The general swelling of the cells was even more noticeable than in the liver. Similar results were obtained with the cells of the parotid gland.

Figs. 9 and 10 illustrate the results of a fixation experiment on the jejunum of a 15 mm. rat embryo. In the case of the intestinal epithelium, as in the other examples, the main difference appeared to reside in the vacuolization and precipitation of the ground substance induced by unbuffered OsO_4. The precipitate blurred the structural details of the particulate components of the cytoplasm, which, in addition, seemed to clump together in irregular aggregates. Similar, though less pronounced differences, were obtained with kidney cells of adult and new born rats.

In the case of pancreas, intestine, and kidney, the intermediary, useful zone of the blocks appeared to be much wider than in the case of the liver. Correspondingly, the central zone, which showed in general a fixation of poor quality, was much reduced in size, or absent. This difference is probably due to the presence in the organs first mentioned, of much loose, interstitial connective tissue, which favors a more rapid diffusion of the fixative throughout the tissue block.

DISCUSSION

One of the most difficult problems in electron microscope cytology is to evaluate the quality of fixation; *i.e.*, to decide how closely the fixed image ap-

proximates that of the living cell. Obviously, this is due to the fact that living material cannot be studied at resolutions better than 0.2 to 0.1 μ. The problem is further magnified by the fact that in the electron microscope the appearance of fixed cells varies appreciably with the fixative employed (5–7, 9, 26). The experiments reported in the present study demonstrate that even when a single fixative, *e.g.* OsO_4, is used, the morphology of the cells is influenced by secondary factors; *e.g.*, the H^+ activity of the fixative solutions. Preliminary observations seem to indicate that the over-all molarity of these solutions also has noticeable influence.

At the beginning of this study, it was assumed that the appearance in the electron microscope of tissue culture cells fixed by OsO_4 vapors closely approximates that of living cells. This assumption finds support from the following considerations:

(*a*) It has been known since Strangeways and Canti's study (27) that fixation by OsO_4 does not cause any morphological alterations in cultured cells. The latter were observed under the dark field microscope during exposure to OsO_4 and it was found that the cellular components visible in the light microscope retained their morphology, while the ground substance of the cytoplasm as well as the nuclear content showed only a slight increase in light-scattering power. In the cytoplasm, the increase was much fainter towards the periphery of the cell, a region preferentially used for electron microscope studies. It appears established therefore, that OsO_4 fixation of tissue culture cells does not produce morphological changes above the limit of resolution of the light microscope. It may be assumed that the same obtains, to a certain extent at least, below this limit, as indeed Strangeways and Canti's observations suggest. The mentioned increase in light-scattering power indicates that a change in the state of dispersion, in the sense of a higher degree of aggregation, occurs during OsO_4 fixation both in the nuclear content and in the ground substance of the cytoplasm. This change could take place at a very low level, without necessarily interfering with the fine structure of the cell, because the dark field microscope is supposed to be able to detect particles as small as 0.006 μ (28).

(*b*) Below the limit of resolution of the light microscope, cultured cells fixed in OsO_4 show an "endoplasmic reticulum" embedded in a continuous and frequently homogeneous matrix (5, 8, 29). Porter recently presented evidence supporting the existence of the endoplasmic reticulum *in vivo* (29).

(*c*) As for the matrix of the ground substance, its homogeneous appearance is in agreement with current ideas concerning the physical state of the ground substance which may exist as a sol or as a gel with "submicroscopic" (by reference to the electron microscope) structure (30). With these considerations in mind, it is reasonable to assume that tissue culture cells retain, after OsO_4 fixation, a morphology very similar to the one they had in the living state. Taken at its least, it represents the best approximation thus far obtained in fixed material.

Because of the facts just stated, the appearance of tissue culture cells fixed in OsO_4 vapors was taken as a standard of reference, and the results obtained in the present experiments were deemed good or bad as judged by it. As already

indicated, the standard can be approached or duplicated in cells of tissue blocks fixed with OsO_4 solutions buffered at pH 7.3–7.5. Comparable results were obtained with acetate-veronal and phosphate buffer, although the former seems to be better. Other buffers will have to be tested in the future, as the pH of the fixative seems to be more important than the nature of the buffer system. In this respect it may be pointed out that in general cultured cells are fixed while immersed in a balanced salt solution (*e.g.*, Tyrode's or Earle's) buffered at a slightly alkaline pH (between 7.0 and 7.6). This, together with the very shallow depth the fixative has to penetrate in these thinly spread cells, may explain the good results obtained with tissue culture material.

It is not intended to compare the fine structure details previously described in cultured cells (5, 8) with those now found in cells fixed in tissue blocks. The nature of the canalicular structures found in the ground substance of the cytoplasm will be dealt with in a following paper. The cytoplasmic matrix of cultured, mesothelial cells occasionally shows bundles of intracellular fibrils. No similar appearances have been found thus far, in the epithelial cells of tissue blocks. This does not mean that buffered OsO_4 is not capable of fixing intracellular fibrils; for fine fibrillar structures were found to be well preserved by buffered OsO_4 in specialized cells; *e.g.*, heart and smooth muscle fibers and epidermal epithelia.

The influence of the H^+ activity of the fixing solutions upon the appearance of fixed cells has been studied by light microscopists, especially by botanists (Yamata, Zirkle). A discussion of the problem can be found in Zeiger (31). The general conclusion of these studies was that a good fixative should have a pH of 4.0–5.0. This does not agree with the present findings, but it has to be pointed out that in the studies cited, the material, the point of main interest, and the microscope in particular, were different.

The use of a buffered fixative for ordinary microscopical preparations was proposed by Gross and Lohaus (32) in 1932. It was a formaldehyde-$CaCl_2$-K_2O_4Cr mixture buffered at pH 7.2 with borate buffer. This formula was the result of a series of experiments aimed at finding a fixative compatible with minimal changes in volume, reaction, and N content of tissue blocks. The changes in volume were followed by weighing and the loss in organic material by N determinations in the fixing solutions. No particular interest was directed towards the preservation of the morphological detail and it appears that the proposed fixative has not been accepted for general use.

In electron microscopy, the damaging effects of acid-containing fixatives, both upon cultured cells (5, 6) and upon cells in tissue blocks (9), have already been pointed out. It has also been shown on both kinds of material (6, 33) that neutral solutions of formaldehyde give a better fixation than the usually acid solutions of the same fixative. As far as it can be ascertained, no buffered fixative solution has been utilized until the present study.

The definite effects of various levels of H^+ activity and various buffer sys-

tems upon the morphology of the fixed cell indicate that the cell permeability is seriously altered just prior to or during fixation. Under such conditions it appears reasonable to use a buffered fixative which will maintain the cell content at a close to normal pH during fixation and thus will increase the chances of a life-like preservation of the cell structure.

The acid production during OsO_4 fixation remains unexplained. It emphasizes how little we know about the chemistry of this process, a subject which deserves more attention than is presently given.

SUMMARY

Osmium tetroxide fixation of tissue blocks, as usually effected, is preceded by an acidification of the tissue. This acidification is probably responsible for morphological alterations which are notably disturbing in electron microscopy. The acidification and the resulting morphological alterations cannot be prevented by homogenizing the tissue directly in OsO_4 solutions or by adding enzyme inhibitors (fluoride, iodoacetamide) to the fixative.

Fixation experiments with buffered OsO_4 solutions have shown that the appearance of the fixed cells is conditioned by the pH of the fixative. The quality of fixation can be materially improved by buffering the OsO_4 solutions at pH 7.3–7.5. The acetate-veronal buffer appeared to be the most favorable of the buffers tested. Because of these findings, 1 per cent OsO_4 buffered at pH 7.3–7.5 with acetate-veronal buffer is recommended as an appropriate fixative for electron microscopy.

BIBLIOGRAPHY

1. Pease, D. C., and Baker, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 470.
2. Geren, B. B., and McCullock, D., *Exp. Cell. Research*, 1951, **2**, 97.
3. Newman, S. B., Borisko, E., and Swerdlow, M., *J. Research Nat. Bureau Stand.*, 1943, **43**, 183.
4. Latta, H., and Hartmann, F. J., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 436.
5. Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.
6. Porter, K. R., *Anat. Rec.*, 1950, **106**, 145.
7. Barer, R., Cytological Techniques, *in* Cytology and Cell Physiology, (G. Bourne, editor), Oxford, Clarendon Press, 2nd edition, 1951.
8. Porter, K. R., and Kallman, F. L., *Exp. Cell. Research*, in press.
9. Dalton, A. J., Kahler, H., Striebich, M. J., and Lloyd, B., *J. Nat. Cancer Inst.*, 1950, **11**, 439.
10. Michaelis, L., *Biochem. Z.*, 1931, **234**, 139.
11. Claude, A., *Harvey Lectures*, 1947–48, **43**, 121.
12. Hillier, J., and Gettner, M. D., *Science*, 1950, **112**, 520.
13. du Pont de Nemours and Co., *Ind. and Eng. Chem.*, 1936, **28**, 1161.
14. Anderson, T. F., *Tr. New York Acad. Sc.*, 1951, **13**, 130.
15. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.
16. Palade, G. E., and Claude, A., *J. Morphol.*, 1949, **85**, 71.

17. Baker, J. R., Cytological Technique, London, Methuen & Co. Ltd., 2nd edition, 1945, 82–83.
18. Moore, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1917, **15**, 18.
19. Chambers, R., and Pollack, H., *J. Gen. Physiol.*, 1927, **10**, 739.
20. Chambers, R., *Biol. Symposia*, 1943, **10**, 91.
21. Parat, M., *Arch. anat. micr.*, 1928, **24**, 73, 297.
22. Rous, P., *J. Exp. Med.*, 1925, **41**, 739.
23. Schmidtmann, M., *Z. ges. exp. Med.*, 1925, **45**, 716.
24. McIlvaine, T. C., *J. Biol. Chem.*, 1921, **49**, 183.
25. Palade, G. E., and Claude, A., *J. Morphol.*, 1949, **85**, 35.
26. Bretschneider, L. H., *Proc. K. Nederl. Acad. Wetenschap.*, 1950, **53**, 675.
27. Strangeways, T. S. P., and Canti, R. G., *Quart. J. Micr. Sc.*, 1927, **71**, 1.
28. Zsigmondy, R., Kolloid Chemie, Leipzig, 1925, cited by Frey-Wyssling (30, p. 9).
29. Porter, K. R., *Ann. New York Acad. Sc.*, in press.
30. Frey-Wyssling A., Submicroscopic Morphology of Cytoplasm and Its Derivatives, New York, Elsevier Publishing Co. Inc., 1948.
31. Zeiger, K., Physikochemische Grundlagen der histologischen Metodik, Dresden and Leipzig, Steinkopff, 1938.
32. Gross, W., and Lohaus, H., *Z. wissensch. Mikr.*, 1932, **49**, 168.
33. Rozsa, G., and Wyckoff, R. W. G., *Biochem. et Biophysic. Acta.*, 1950, **6**, 334.

EXPLANATION OF PLATES

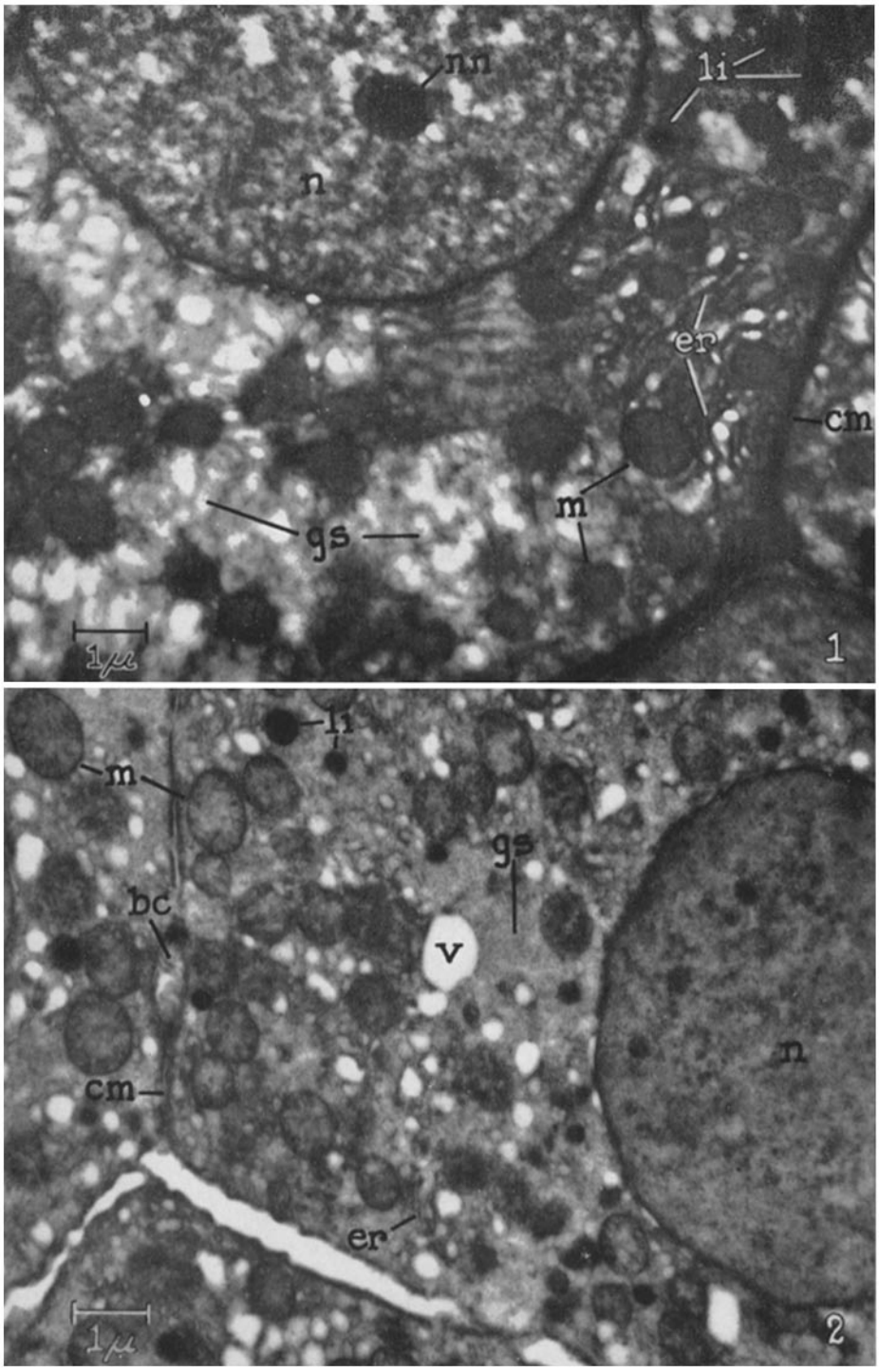
PLATES 19 AND 20

FIGS. 1 to 4. Electron micrographs showing sections of parenchymal cells obtained from the liver of the same rat. The blocks were fixed in 1 per cent OsO_4 buffered with acetate-veronal buffer at the following pH's: pH 5.0 for block No. 1 (Fig. 1); pH 6.0 for block No. 2; pH 7.0 for block No. 3; and pH 8.0 for block No. 4. The rest of the processing (dehydrating, embedding, sectioning) was the same for all four blocks.

PLATE 19

FIGS. 1 and 2. Only a part of a liver cell enlarged 10,000 × is shown in each figure. In Fig. 1, half of a section of a nucleus (*n*) appears in the upper part of the picture; in Fig. 2, more than half of the nuclear section (*n*) can be seen along the right side of the picture. A comparative description of the two figures follows:

The nuclear content shows a coarse reticular precipitation in Fig. 1 and a much finer one in Fig. 2. A nucleolus (*nn*), visible only in Fig. 1, appears as a large, dense, almost homogeneous granule. Numerous *mitochondria* (*m*) appear in cross-section in both figures. In Fig. 1 they have a thicker membrane and a denser content. *The lipid inclusions* (*li*) are difficult to distinguish from the mitochondria in Fig. 1, in which a few can be found in the upper right corner. They are more easily distinguished in Fig. 2 in which they appear smaller, denser, and more homogeneous than the mitochondria. In Fig. 1 (pH 5.0), the *ground substances of the cytoplasm* (*gs*) is precipitated in a coarse network especially visible in the left half of the picture; in Fig. 2 (pH 6.0), it shows as a fine reticular or granular precipitate and, in addition, contains a large number of vacuoles (*v*) of various sizes, most of which do not appear to have a clearly differentiated membrane. In Fig. 1, a group of elongated structures (*er*) separated by numerous vacuoles can be found embedded in the ground substance. It is difficult to decide whether they have a filamentous or canalicular structure. Similar entities (*er*) but fewer and with a more definite canalicular character, are to be found in Fig. 2. The *cell membranes* (*cm*) are thicker and denser in Fig. 1; in Fig. 2, the corner of the lower cell is separated by an open space from the two upper ones. No such intercellular spaces were observed at more acid pH's. Along the vertical intercellular border, visible in the left half of Fig. 2, there can be seen a section through a bile capillary (*bc*) in which are what look like protruding papillae.



(Palade: Study of fixation for electron microscopy)

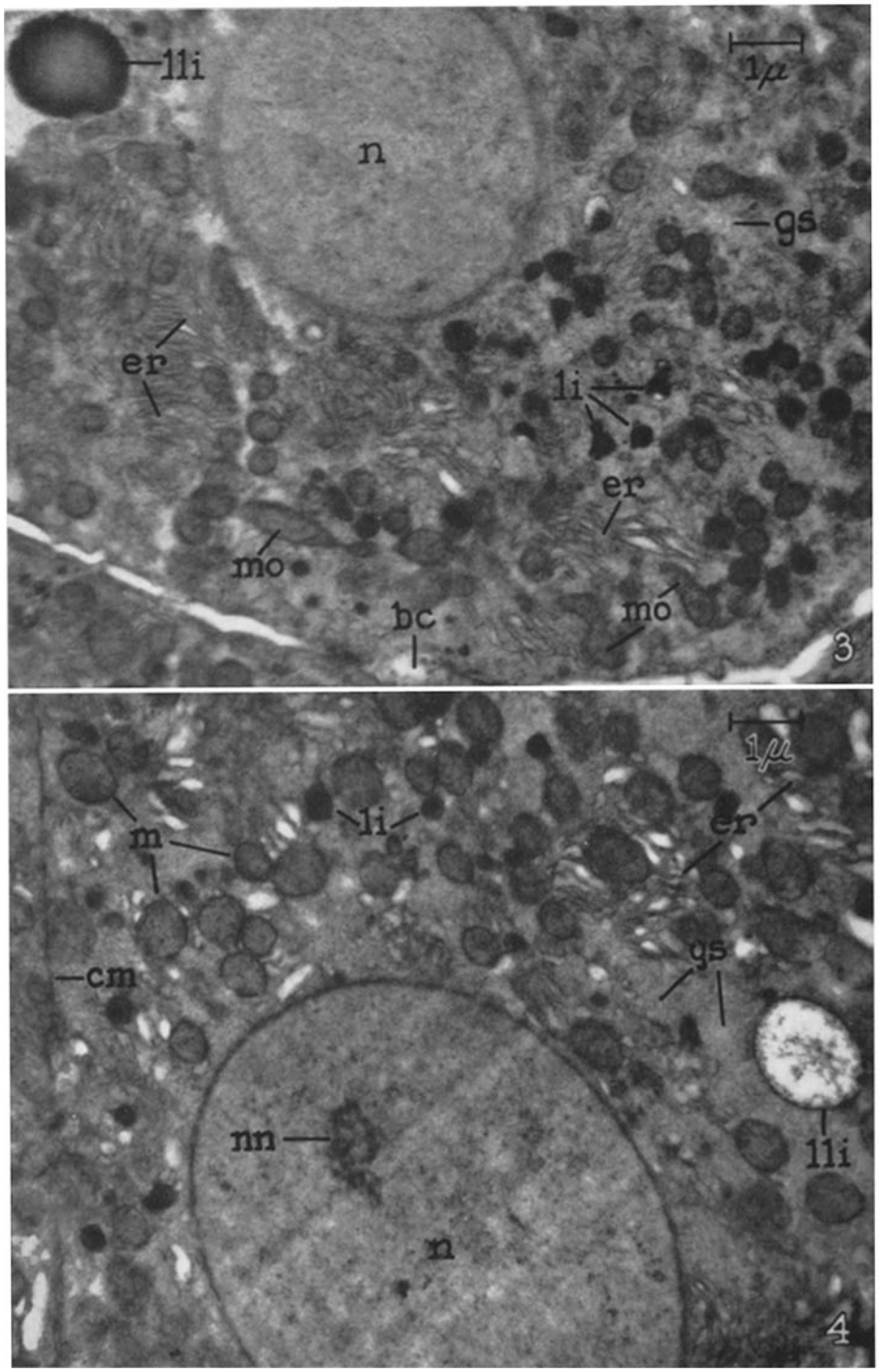
PLATE 20

FIGS. 3 and 4. For general explanation read explanation of Figs. 1 to 4.

Fig. 3 (Fixation: 1 per cent OsO_4 buffered at pH 7.0) shows part of a liver cell section which cuts tangentially through the nucleus. The latter (*n*) is visible in the center of the upper half of the picture; its obviously thick membrane has resulted from the tangential orientation of the section. Fig. 4 (Fixation: 1 per cent OsO_4 buffered at pH 8.0) shows part of a liver cell section which has cut more deeply into the nucleus (*n*), visible in the center of the lower half of the picture. For both figures the enlargement is 10,000 ×. A description in parallel of Figs. 3 and 4 follows:

The nuclear content is almost homogeneous in Fig. 3 and less so in Fig. 4, in which in addition to a dense nucleolus (*nn*) which appears convoluted, some other denser aggregates are scattered throughout a finely granular background. *The mitochondria* have smaller diameters in Fig. 3, in which a number of them appear in oblique section (*mo*). In Fig. 4, the mitochondria (*m*) show larger diameters suggestive of a certain amount of swelling. The *lipid inclusions* (*li*) can be easily recognized in both cases because of their high density. The smaller of them frequently show little vacuoles, central or superficial. A large lipid inclusion (*lli*) in the upper left corner of Fig. 3 has a homogeneous appearance. A similar inclusion (*lli*) appears to have been replaced by a large vacuole with precipitated content at pH 8.0 (Fig. 4). At the same pH the small lipid inclusions show less vacuolization than at pH 7.0. *The ground substance of the cytoplasm* shows, in both cases, arrays of more or less parallel structures (*er*) which seem to be of a canalicular character. More vacuoles are present in between these structures in Fig. 4 than in Fig. 3. The matrix of the ground substance (*gs*) is almost homogeneous in both cases but shows more vacuoles in Fig. 4. *Cell margins* can be seen in the lower corners of Fig. 3. Along them, at *bc*, the lumen of a bile capillary is shown in part. A cell boundary (*cm*) is evident along the left side of Fig. 4. In both cases, the cell membranes appear thin and not very dense.

Of the cells shown in Figs. 1 to 4, the one reproduced in Fig. 3, *i.e.* that which was fixed at pH 7.0, comes closest in its appearance to a tissue culture cell fixed by OsO_4 vapors. For this reason, it is judged to represent the best quality of fixation obtained in this pH series.



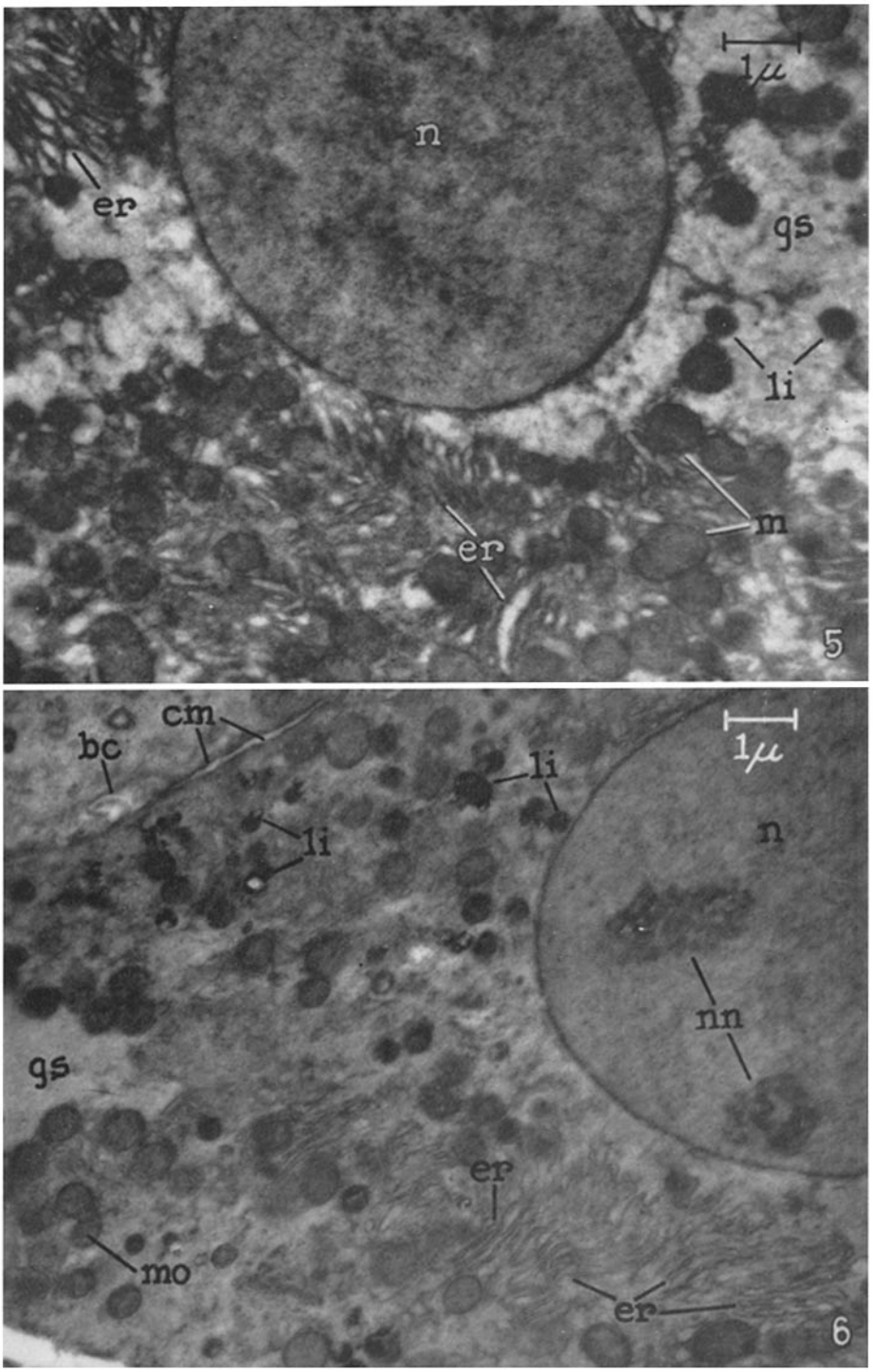
(Palade: Study of fixation for electron microscopy)

PLATE 21

FIGS. 5 and 6. Electron micrographs showing sections through rat liver cells obtained from the same animal. The processing of the liver blocks was the same, except that the block for Fig. 5 was fixed in 1 per cent OsO_4 in distilled water, while that for Fig. 6 was fixed in 1 per cent OsO_4 buffered at pH 7.3 with acetate-veronal buffer.

In both figures, only a part of a cell section is shown, enlarged 10,000 ×. Almost the entire section of a nucleus (*n*) appears in Fig. 5, while half of the section of a nucleus (*n*) is visible in Fig. 6. Both nuclei show what looks like a double membrane. This appearance is probably due to the sphericity of the nuclei and to the thickness of the sections.

The *nuclear content* is definitely not homogeneous in Fig. 5; it appears more homogeneous in Fig. 6, which also shows two nucleoli (*nn*) of convoluted structure. *The mitochondria* are mostly cut in cross-section, but a few have been cut on the bias as can be seen in Fig. 6 (*mo*). In Fig. 5, the diameter of the mitochondria is larger than in Fig. 6. This probably indicates a swelling of these organelles in unbuffered OsO_4. *The lipid inclusions* (*li*) are difficult to distinguish from the mitochondria in Fig. 5. They can, however, be clearly differentiated in Fig. 6, where they are particularly numerous in the upper half of the picture. They are smaller and much denser than the mitochondria, and they frequently show vacuoles, either inside or at their surfaces. *The ground substance* in Fig. 5 shows a number of elongated structures (*er*), most of them filamentous and interspersed with vacuoles, throughout the lower half of the picture; a smaller group is visible in the upper left corner. In Fig. 6, the same structures appear to have a definite canalicular character. They lie parallel with almost no vacuolization between them. A large aggregate of such canaliculi (*er*) can be seen in the lower half of Fig. 6. The matrix of the ground substance (*gs*) shows a coarse precipitation in Fig. 5, whereas in Fig. 6 it appears relatively homogeneous. Note the difference in its electron scattering power between Fig. 5 (lower density) and Fig. 6 (higher). No cell margin is visible in Fig. 5. Cell membranes (*cm*) appear in the upper left corner of Fig. 6; note in between the cells, the oblong section through a bile capillary (*bc*) with what look like filiform papillae protruding into it.



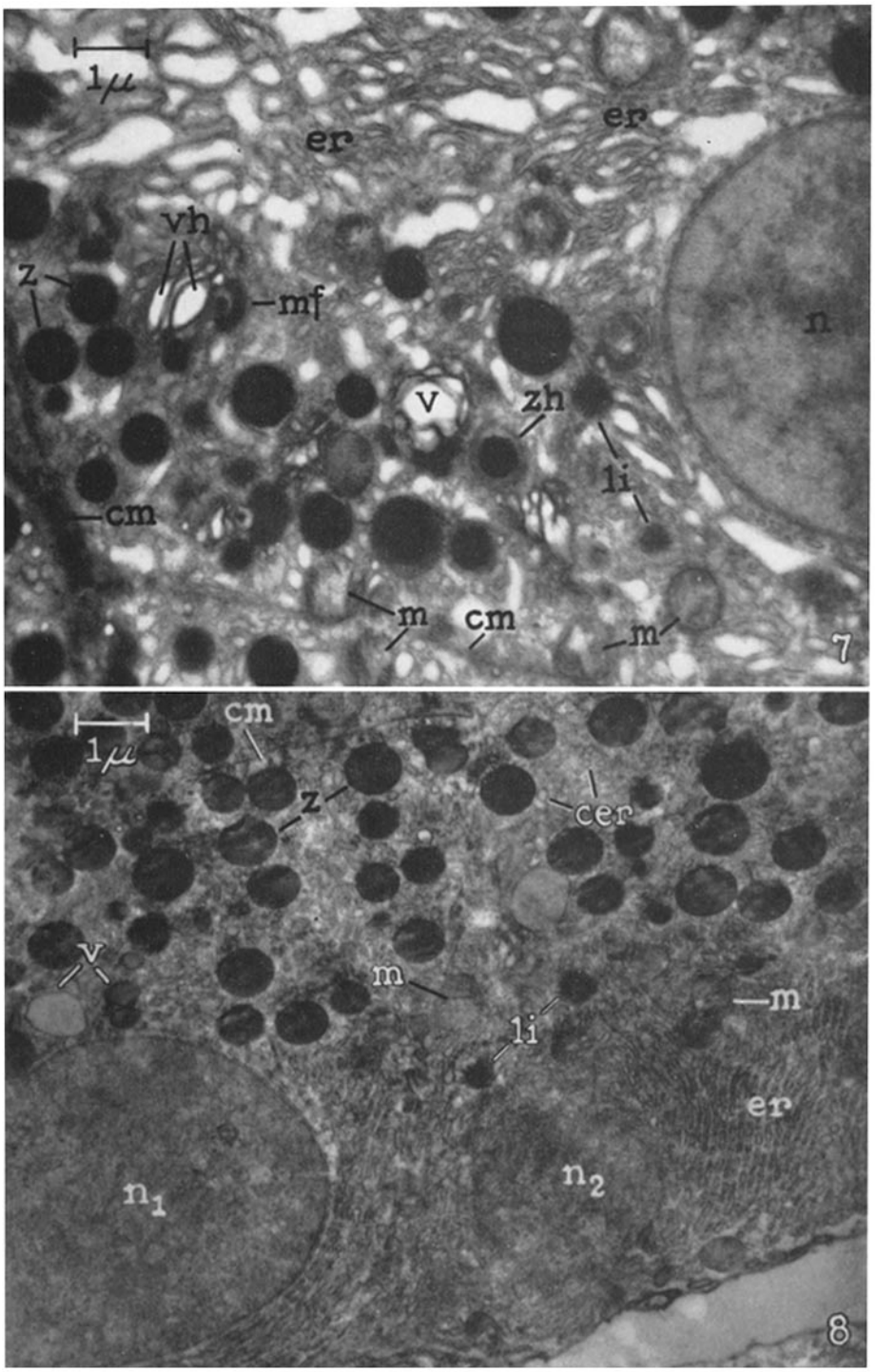
(Palade: Study of fixation for electron microscopy)

PLATE 22

FIGS. 7 and 8 are electron micrographs of pancreatic exocrine cells obtained from the same animal. The processing of the blocks was the same, except that the block for Fig. 7 was fixed in 1 per cent OsO_4 in distilled water, whereas that for Fig. 8 was fixed in 1 per cent OsO_4 buffered at pH 7.3 with acetate-veronal buffer. Both photomicrographs are enlarged 10,000 ×.

In Fig. 7, only part of the apical pole of a cell is shown. Part of the nucleus (*n*) appears on the right side of the picture, and cell boundaries (*cm*) can be seen along the lower and the left-hand sides. In Fig. 8, parts of two adjacent cells are shown. The limit of the acinus can be seen in the lower right corner. The nucleus of a cell (n_1 appears in the lower left corner; the nucleus of another (n_2) is just included in the section. A comparative description of Figs. 7 and 8 follows:

The nuclear content shows a reticular precipitation in Fig. 7, but appears more homogeneous in Fig. 8. The zymogen granules (*z*) appear as uniformly dense, circular spots in the apical pole of the cells. They seem to be swollen and not very well preserved in Fig. 7, in which some of them show a diffuse halo. This is not so in Fig. 8. The *lipid inclusions* (*li*) cannot with certainty be distinguished from the zymogen granules. They seem to be smaller, almost as dense as the latter, but less sharply outlined. In Fig. 7, some of them appear to be present to the left of the nucleus and in the lower left corner of the cell. In Fig. 8, similar bodies appear on the apical side of the nucleus to the right. Some vacuoles (*v*) can be seen in the apical region of the cells; they are large, multiloculated, with thick, laminated walls, in Fig. 7. This last feature together with such appearances as the dense "hook" (*mf*) next to the vacuole identified by *vh* are highly reminiscent of myelin figures. No corresponding structures can be found in Fig. 8, in which only relatively small vacuoles (*v*) with light or dark content can be seen to the left, immediately above the nucleus. *The mitochondria* (*m*) are scarce in general; in Fig. 7, they appear swollen and partially disrupted, whereas in Fig. 8 they seem better preserved. *The ground substance* in Fig. 7 shows a reticulum of filamentous or canalicular structures (*er*) separated by large, empty, irregular spaces or vacuoles. The same entities appear in impressive abundance in Fig. 8, in which their canalicular aspect shows more clearly. They lie next to one another in whorls around the nuclei and the cell particulates. At the basal pole of the cells they appear in longitudinal (*er*) and at the apical pole in cross-section (*cer*). The cell boundaries (*cm*) appear thick and fuzzier in Fig. 7; thin and in some places sharply outlined in Fig. 8.



(Palade: Study of fixation for electron microscopy)

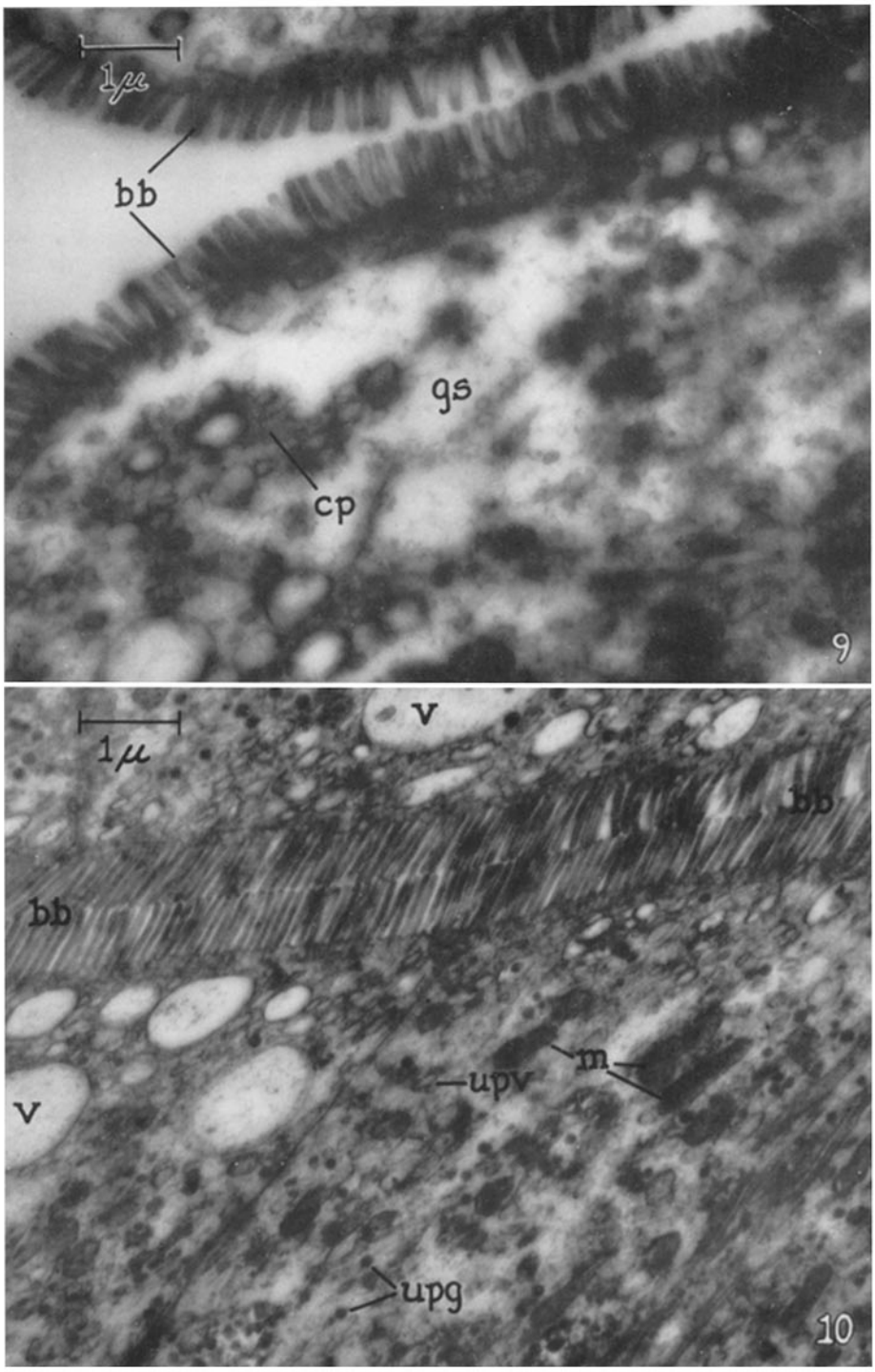
PLATE 23

FIGS. 9 and 10. Electron micrographs showing partial views of epithelial cells of rat embryo intestinal mucosa (jejunum). For both, the magnification is 13,000 ×. The blocks were obtained from the same embryo and processed in the same way, except that that for Fig. 9 was fixed in 1 per cent OsO_4 in distilled water, whereas that for Fig. 10 was fixed in 1 per cent OsO_4 buffered at pH 7.3 with acetate-veronal buffer.

Fig. 9 was taken at the level of a fold in the epithelial layer, as shown by the disposition of the brush border (*bb*). Only the apical pole of a few cells is shown in the picture; the nuclei and the basal poles are completely out of the field.

Fig. 10 reproduces a small field of a section through two villi, so closely packed that their brush borders are almost "zippered" together. These borders appear as two bands that go across the picture from *bb* to *bb*. As in Fig. 9, only the apical poles of the epithelial cells are included in the picture. A comparative description of the two pictures follows.

There is no obvious difference in the state of preservation of the brush borders. The *mitochondria* seem to be swollen and are difficult to identify in Fig. 9, whereas in Fig. 10 (*m*) they appear to be better preserved. *The ground substance of the cytoplasm* (*gs*) in Fig. 9 shows a coarse precipitation of its matrix with many large spaces and vacuoles. Although in Fig. 10 a number of vacuoles (*v*) are present immediately below the brush border, the rest of the ground substance is free from them and does not show the same amount of precipitation as in Fig. 9. There are numerous, unidentified particles, both granular (*upg*) and vesicular (*upv*) in character, in the cytoplasm of Fig. 1 The corresponding elements are not found, or are difficult to identify in cells fixed in unbuffered OsO_4 (Fig. 9). In these latter there is a marked tendency of the smaller particulates to aggregate in clumps (*cp*) separated by expanses of precipitated ground substance. The cell margins in Fig. 10 are sharp. No cell boundaries are seen in Fig. 9, but in unbuffered OsO_4 they appear in general less distinct than in buffered OsO_4.



(Palade: Study of fixation for electron microscopy)