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CYTOCHEMISTRY AND ELECTRON MICROSCOPY

The Preservation of Cellular Ultrastructure and Enzymatic Activity by Aldehyde Fixation

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ABSTRACT

The aldehydes introduced in this paper and the more appropriate concentrations for their general use as fixatives are: 4 to 6.5 per cent glutaraldehyde, 4 per cent glyoxal, 12.5 per cent hydroxyadipaldehyde, 10 per cent crotonaldehyde, 5 per cent pyruvic aldehyde, 10 per cent acetaldehyde, and 5 per cent methacrolein. These were prepared as cacodylate- or phosphate-buffered solutions (0.1 to 0.2 M, pH 6.5 to 7.6) that, with the exception of glutaraldehyde, contained sucrose (0.22 to 0.55 M). After fixation of from 0.5 hour to 24 hours, the blocks were stored in cold (4°C) buffer (0.1 M) plus sucrose (0.22 M). This material was used for enzyme histochemistry, for electron microscopy (both with and without a second fixation with 1 or 2 per cent osmium tetroxide) after Epon embedding, and for the combination of the two techniques. After fixation in aldehyde, membranous differentiations of the cell were not apparent and the nuclear structure differed from that commonly observed with osmium tetroxide. A postfixation in osmium tetroxide, even after long periods of storage, developed an image that—notable in the case of glutaraldehyde—was largely indistinguishable from that of tissues fixed under optimal conditions with osmium tetroxide alone. Aliesterase, acetylcholinesterase, alkaline phosphatase, acid phosphatase, 5-nucleotidase, adenosine triphosphatase, and DPNH and TPNH diaphorase activities were demonstrable histochemically after most of the fixatives. Cytochrome oxidase, succinic dehydrogenase, and glucose-6-phosphatase were retained after hydroxyaldipaldehyde and, to a lesser extent, after glyoxal fixation. The final product of the activity of several of the above-mentioned enzymes was localized in relation to the fine structure. For this purpose the double fixation procedure was used, selecting in each case the appropriate aldehyde.

In this paper seven different organic substances are introduced as fixatives for electron microscopy and cytochemistry. The work, as a means of satisfying the requirements of each, has taken into consideration the difficulties experienced when combining the two fields (1, 2). At the present time, osmium tetroxide (3) and, to a lesser extent, potassium permanganate (4) are the fixatives of choice that are currently used in morphological studies with the electron microscope. These reagents provide excellent cytological fixation, and also add density to some of the reactive components of the cells. However, their use in cytochemistry is very limited since they are heavy metal–containing, oxidizing reagents that completely destroy enzymatic activities except after very short periods of fixation (5–9).

Because of this circumstance, previous work in

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the application of enzyme cytochemistry to electron microscopy has dealt primarily with incubation of fresh (10, 11) or formalin-fixed tissue (12–14) followed by postincubation fixation in osmium tetroxide. These methods are only partly adequate as a compromise. In our experience the disruption caused by incubation of fresh tissues was usually severe and the preservation attained by preincubation fixation in formalin not always satisfactory. Acrolein (15), which also requires postfixation in osmium tetroxide, preserves the submicroscopic organization of cells more adequately, but its extreme reactivity largely accounts for a rather complete destruction of enzymatic activities and therefore seriously limits its potential use in enzyme histochemistry.

It occurred to us that an acceptable compromise might be accomplished with aldehydes as fixatives. Glyoxal, glutaraldehyde, and hydroxyadipaldehyde[1] are effective cross-linking agents for proteins and polyhydroxy compounds (16–20) and were therefore studied first. The results were then compared with those given by acrolein and formalin, and subsequently the monoaldehydes crotonaldehyde, methacrolein, acetaldehyde, and pyruvic aldehyde[1] were also studied. These reagents were studied as fixatives for electron microscopy after we knew that most of them retained a variety of enzymatic activities in several tissues as demonstrated by histochemical methods at the light microscope level. Attention was paid in the present study to the following variables encountered in fixation: concentration of the reagents, duration of fixation, and osmolarity and pH of the fixing fluid. Since in solution the majority of these fixatives are acidic, the effects of different buffers at several pH's were noted.

As has been shown with formalin (21), when organic fixatives are used it is convenient to divide the process of fixation for cytochemistry with the electron microscope into two parts. Initially, fine structure as well as enzymatic activity is preserved by means of the aldehyde; in a second step, the action of osmium tetroxide allows the relation to be made between the final product of the histochemical test and the fine structure as it is now accepted. The latter fixation also provides the necessary contrast in the electron image. The reactions for the localization of enzymatic activity are performed between the two fixations.

[1] Obtained through the courtesy of Union Carbide and Chemical Co.

As this procedure was the one adopted when the previously mentioned aldehydes were used for histochemical purposes, we shall analyze our results in two parts. The first is concerned with the fine structural details of tissues fixed in one or another of the aldehydes alone or fixed in an aldehyde and refixed in osmium tetroxide. The second part shows that the combination of cytochemistry and electron microscopy can be easily and profitably accomplished with tissues fixed in several aldehydes. This part deals with the localization of various enzymatic activities (some of which have not heretofore survived fixation) with the light and the electron microscope. These results have been reported very briefly before (22, 23).

MATERIAL AND METHODS

Aldehyde Fixation

In preliminary studies we used sections of the aldehyde-fixed paraffin-embedded blocks successfully for routine histology with hematoxylin and eosin (Fig. 1) or several trichrome stains.

Optimal conditions of fixation with each aldehyde for electron microscopy were then established and are summarized in Table I. It will be noted that in some cases (glyoxal and pyruvic aldehyde) the final pH of the fixing solution was acidic. No further neutralization was attempted, in order to avoid a loss of aldehyde concentration through a Cannizzaro type of reaction. Amine-containing buffers such as tris(hydroxymethyl)aminomethane were avoided because they would be expected to react with aldehydes. In all cases the fixative solutions were hypertonic. For instance, 6.5 per cent glutaraldehyde in 0.1 M phosphate buffer was 980 milliosmols (as determined cryoscopically). With this concentration some shrinkage was observed, especially when free cells were fixed (tissue culture suspension). In this case the aldehyde concentration was reduced to 2 to 3 per cent. Similar observations were made with the other aldehydes.

Although Table I refers to the quality of fixation obtained with rat liver as a test object, other tissues also were used in the morphological studies, including pancreas, kidney, adrenal, peripheral nerve, skeletal and cardiac muscle, testis, and retina from either rat, mouse, hamster, or toad. In some cases, human material also was used. After fixation, the blocks (maximum thickness 0.4 cm) were washed or stored at 4°C in a 0.2 M sucrose solution (buffered at pH 7.4 with 0.1 M phosphate or cacodylate) for varying periods of time, from several hours to several months. After fixation and washing, tissue blocks (1 mm^3), trimmed from the original, were either refixed in 1 or 2 per cent buffered osmium tetroxide containing

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sucrose (24) for 2 to 4 hours, or directly embedded. Alternatively, small blocks (1 mm³) of fresh tissue were fixed directly in the aldehyde, but no major differences were noted in comparison with the previous procedure.

Dehydration of tissue blocks was routinely performed in increasing concentrations of ethanol. Epon embedding (25) was satisfactory and was used for the entire study, when it was found that tissues refixed in osmium tetroxide and embedded in methacrylate suffered severe sublimation under the electron beam. The quality of the morphological preservation was assessed by examination with an RCA EMU-3F electron microscope of thin sections, cut with an LKB ultratome. In order to obtain better contrast, non-osmicated material was usually mounted on naked grids or uncoated Formvar films. Some of the thin sections obtained from a tissue block, fixed only in one or another of the aldehydes and mounted on grids, were stained for different periods of time in solutions of 1 per cent osmium tetroxide or lead hydroxide (26).

TABLE I

Satisfactory Conditions of Fixation for Rat Liver

	Concentration	Buffer	Final molarity of added sucrose	Fixation time of block 1 mm³	Quality of fixation after post-osmication	Final pH
	%			hr.		
Glyoxal	4	0.2 M phosphate or cacodylate	0.22–0.33	2–4	B	6.5
Glutaraldehyde	4–6.5	0.1 M " " "	*	0.5–2	A	7.2
Hydroxy adipaldehyde	12.5	0.1 M " " "	0.44	2–6	C–D	7.5
Crotonaldehyde	10	0.1 M " " "	0.44	2–6	B	7.4
Pyruvic aldehyde	5	0.2 M " " "	0.44	2–6	E	5.5
Acetaldehyde	10	0.1 M " " "	0.22–0.33	2–6	E	7.5
Acrolein	10	0.1 M " " "	*	0.5–2	A	7.6
Methacrolein	5‡	0.1 M " " "	0.22–0.33	2–4	B	7.6
Formaldehyde	4	0.1 M " " "	0.22–0.44	2–4	C	7.4

* No sucrose.

‡ Saturated solution.

Cytochemical Experiments

A variety of tissues (liver, kidney, heart and diaphragmatic muscle, and, to a lesser extent, retina, intestine, and adrenal) of mainly adult albino rats were used as the experimental material. A histochemical survey was first made with the light microscopy of the effects on the enzymatic activity of different times of fixation in various aldehydes. Tissues were fixed in the aldehydes from 2 to 24 hours and then washed and stored in the cold buffer containing sucrose for varying periods of time (hours to weeks). Thin (10 μ) and thick (50 μ) frozen sections were cut and similarly stored, or stained immediately. It should be noted that it was somewhat difficult to prepare thin frozen sections of material fixed for even 6 hours in acetaldehyde, pyruvic aldehyde, glyoxal, and hydroxyadipaldehyde.

For subsequent electron microscopy, tissues were fixed for 10 to 30 minutes in acrolein, or for 2 to 6 hours in the other fixatives, and then washed and stored as described above. In addition, small blocks (1 mm³) were cut from the large blocks used for ordinary frozen sections. The small blocks or thick frozen sections were incubated, along with thin frozen sections, in the various histochemical media. In some reactions (esterases) the material destined for electron microscopy was removed when a discrete initial reaction was observed in the thin frozen sections incubated in the same media. In others (the phosphatases and oxidases), the incubation was allowed to continue for 30 to 60 minutes. After incubation, the thin frozen sections were mounted in glycerine jelly for observation. The small blocks or thick frozen sections were washed briefly in buffer and most of them were refixed in 1 per cent osmium tetroxide for from 15 minutes to 2 hours. These tissues as well as those not postfixed in osmium tetroxide were processed as already described.

The histochemical reactions to demonstrate the following activities were prepared largely according to the original descriptions, and, except when the contrary is indicated, the incubations were performed at room temperature. (*a*) Succinic dehydrogenase ac-

tivity was demonstrated with nitro BT (27) as the reagent. These experiments were performed both aerobically (with and without cyanide in the medium) and anaerobically. Controls included omission of substrate (succinate), and inclusion of malonate (0.1 M) in the medium. (*b*) DPNH and TPNH diaphorase activities were demonstrated with nitro BT as reagent, and DPNH or TPNH respectively as substrate (28). (*c*) Cytochrome oxidase activity was demonstrated by the amine method of Burstone (29). Catalase (0.02 mg/ml) was added to the incubating media to prevent the activity of peroxidase. In some cases the activity of cytochrome oxidase was augmented by addition of cytochrome *c* (0.1 mg/ml) to the medium. Inhibition experiments involved the use of cyanide (10^{-3} M) and sodium azide (10^{-3} M). (*d*) Carboxylic acid esterase activity was demonstrated with a naphthyl acetate as substrate (30) and diazo blue B or garnet GBC as the reagent for light microscope studies. (*e*) In addition, the activity of esterases was also demonstrated with thiolacetic acid as substrate, incubating in the cold (4°C) in the presence of lead ions (9). Separation of the various enzymatic activities able to split thiolacetate was in part obtained by use of inhibitors; for example, activity present at motor end-plates which was sensitive to eserine or DFP (10^{-4} M) was considered acetylcholinesterase. The activity confined to dense bodies (lysosomes) and showing resistance to E 600 (10^{-5} M) (diethyl-*p*-nitrophenyl phosphate) was probably cathepsin C (31). The activity that was resistant to eserine but sensitive to E 600 was considered aliesterase. (*f*) Acid phosphatase activity was demonstrated by the lead nitrate method of Gomori as recently reviewed by Holt (32). In light microscope studies, α-naphthyl phosphate (33) was also used at acid pH in combination with garnet GBC or the hexazonium salt of pararosaniline (34). Inhibition studies used fluoride (10^{-2} M) in the incubating medium. (*g*) Adenosine triphosphatase (ATPase) activity was demonstrated by the method of Wachstein and Meisel (35) and the distribution of this activity was compared with that obtained by substituting glycero-phosphate for ATP in the incubating medium. In light microscope studies the distribution of ATPase activity was compared with that of alkaline phosphatase and of 5-nucleotidase

FIGURES 1 TO 5

Light micrographs of aldehyde-fixed tissues. The histochemical reactions (Figs. 2 to 5) were not counterstained.

FIGURE 1

Human liver tissue (autopsy) fixed in 6.5 per cent glutaraldehyde. Paraffin section stained with hematoxylin and eosin. The spaces between cords of cells are less prominent when lower concentrations of the aldehyde are used. × 560.

FIGURE 2

Frozen section of rat liver fixed in 10 per cent crotonaldehyde and reacted for the demonstration of adenosine triphosphatase (35). × 420.

FIGURE 3

Frozen section of rat liver fixed in 12.5 per cent hydroxyadipaldehyde and reacted for the demonstration of glucose-6-phosphatase activity (40). The diffuse positive reaction appears to be confined to the cytoplasm. Fig. 38 shows the localization in respect to the fine structure. × 460.

FIGURE 4

Glutaraldehyde-fixed rat kidney. Frozen section incubated with thiolacetic acid in the presence of lead ions. The lysosomes of the proximal tubular cells show an intense deposition of lead sulphide. The enzymatic reaction proceeded in the presence of E 600. × 300.

FIGURE 5

Frozen section of hydroxyadipaldehyde-fixed rat myocardium. The dense dots are mitochondrial deposits of the final product of the amine reaction for the demonstration of cytochrome oxidase activity (29). × 520.

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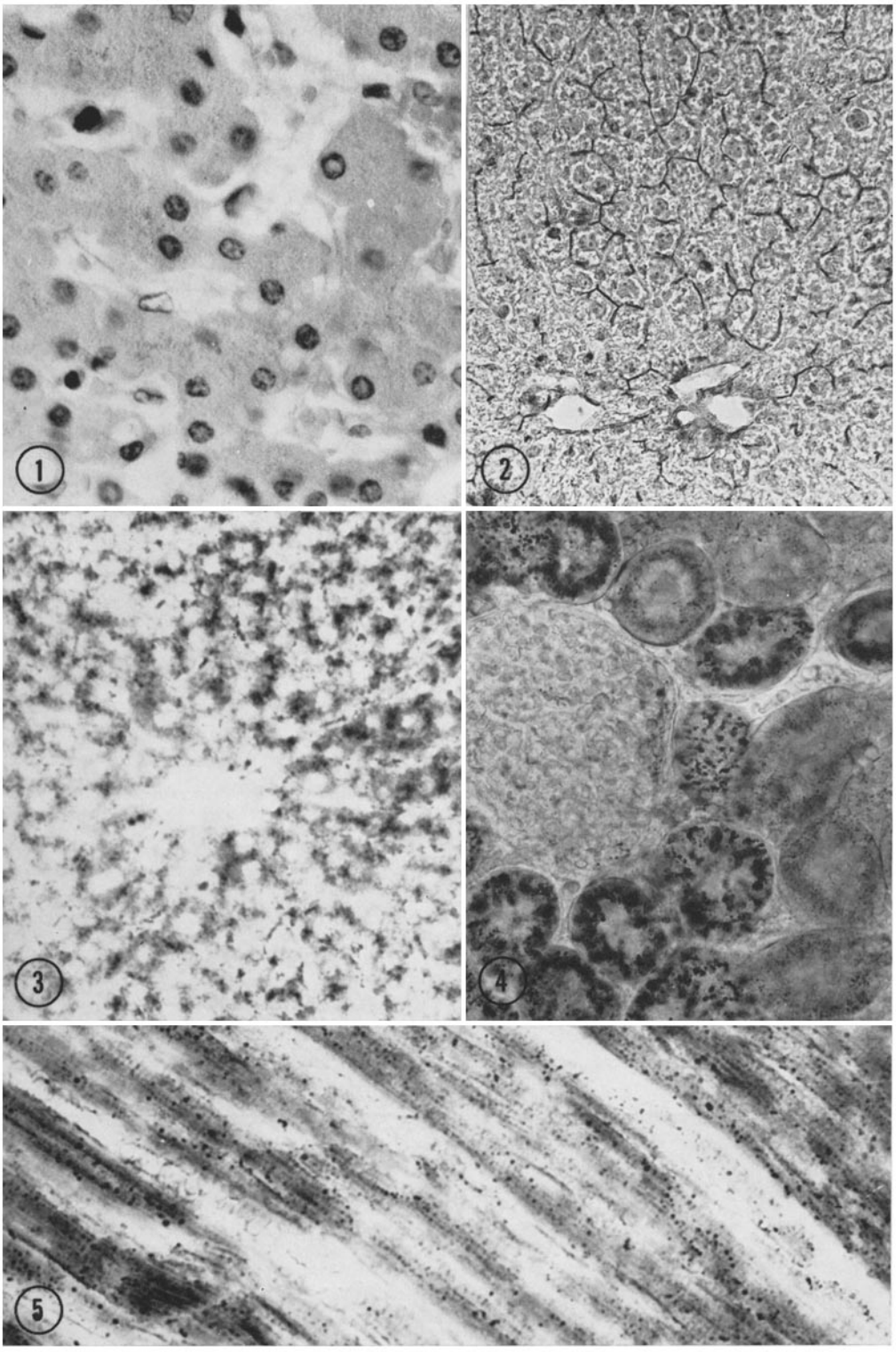

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(36). Alkaline phosphatase activity was demonstrated by the metal salt method of Gomori (37) and an azo dye technique employing naphthyl phosphate (38) with diazo blue B or garnet GBC. Enzymes hydrolyzing nucleotide mono- (36) and diphosphates (39) (muscle adenylic acid (AMP) and inosine diphosphate (IDP)) were localized with the electron microscope in sections incubated with substrate and lead ions. (*h*) Glucose-6-phosphatase activity was demonstrated by the Wachstein and Meisel modification (40) of the Chiquoine method. Control experiments consisted in immersion of sections in acetate buffer, pH 5 (41), which destroyed this enzymatic activity. In all the enzyme studies, frozen sections of fixed materials, immersed in boiling H_2O for several minutes, were also used as controls, which never developed the final product.

RESULTS

Aldehyde Fixation

Fixation in Aldehyde Alone

Thin sections of the aldehyde-fixed material showed a very low contrast in the electron microscope. However, mounting sections on naked grids or uncoated Formvar films made adequate observation possible (Figs. 6 and 7). In general, tissue fixed in aldehydes alone showed a similar nuclear preservation for all fixatives, but the organization of the cytoplasm was clearly better maintained with some aldehydes than with others. In this regard, tissues fixed in glutaraldehyde and acrolein showed excellent morphological preservation which was superior to that obtained with formalin, since swelling and disruption were regularly absent. Glyoxal, methacrolein, and crotonaldehyde showed a preservation at least comparable to that obtained with formalin, but hydroxyadipaldehyde, pyruvic aldehyde, and acetaldehyde generally appeared to be less good fixatives. However, as will be seen later, some of these proved of value in cytochemistry. It should be noted that the quality of preservation of tissues fixed in the aldehydes (especially those of the first group) was found not to be significantly altered by storing in cold buffer containing sucrose for different lengths of time up to several months. This meant that we could obtain a store of large blocks (limiting dimension being a thickness of 0.4 cm) of fixed tissues from which small blocks of thick frozen sections could be cut at any time for electron microscopic study.

Since glutaraldehyde gave the best general preservation of cellular fine structure, the following description will be based mainly on the findings with this fixative. In addition, although a large number of tissues were surveyed, we shall restrict our description mainly to aldehyde-fixed pancreas. This organ was selected not only because the acinar cells are an excellent material for studying cell organelles, but also because a framework of reference is provided by the well known results obtained with osmium tetroxide fixation alone (42, 43) and with the freeze-drying (44, 45) and freeze-substitution techniques (46).

In regard to nuclear structure, we found that chromatin material was not dispersed as is commonly observed in osmium tetroxide–fixed cells, but instead appeared to form compact masses. Some of these occurred free in the karyoplasm, but usually they were applied to the periphery of the nucleus besides surrounding the nucleoli (Figs. 6 to 8). Frequently the chromatin masses were interconnected as well as branched. Within the chromatin masses granules of various sizes (smallest, approximately 100 A) and densities, as well as small ring-shaped profiles, could frequently be discerned (Fig. 6). The interchromatin karyoplasm was composed of a much less dense material in which few granules and some circular or tubular profiles were embedded. The nucleolus appeared as a very finely granular body composed of a material of a density intermediate between the karyoplasm and the chromatin (Figs. 6 to 8). The latter

Figure 6

Partial view of a pancreatic acinar cell fixed in glutaraldehyde alone. The nucleus (*N*) shows the peripheral and perinuclear distribution of chromatin masses (*ch*) and the clear channels (arrows) at the nucleocytoplasmic interface. One of these channels (bottom) extends from the nucleolus (*nu*) beyond the nuclear surface. Others appear to arise in the chromatin masses or in the karyoplasm. In the cytoplasm, ribosomes delineate the general array of the cisternae of the endoplasmic reticulum (*er*). Cristae of mitochondria (*m*) are evident but no outer membranes are visible. Zymogen granules (*z*) are seen at the bottom. × 31,500.

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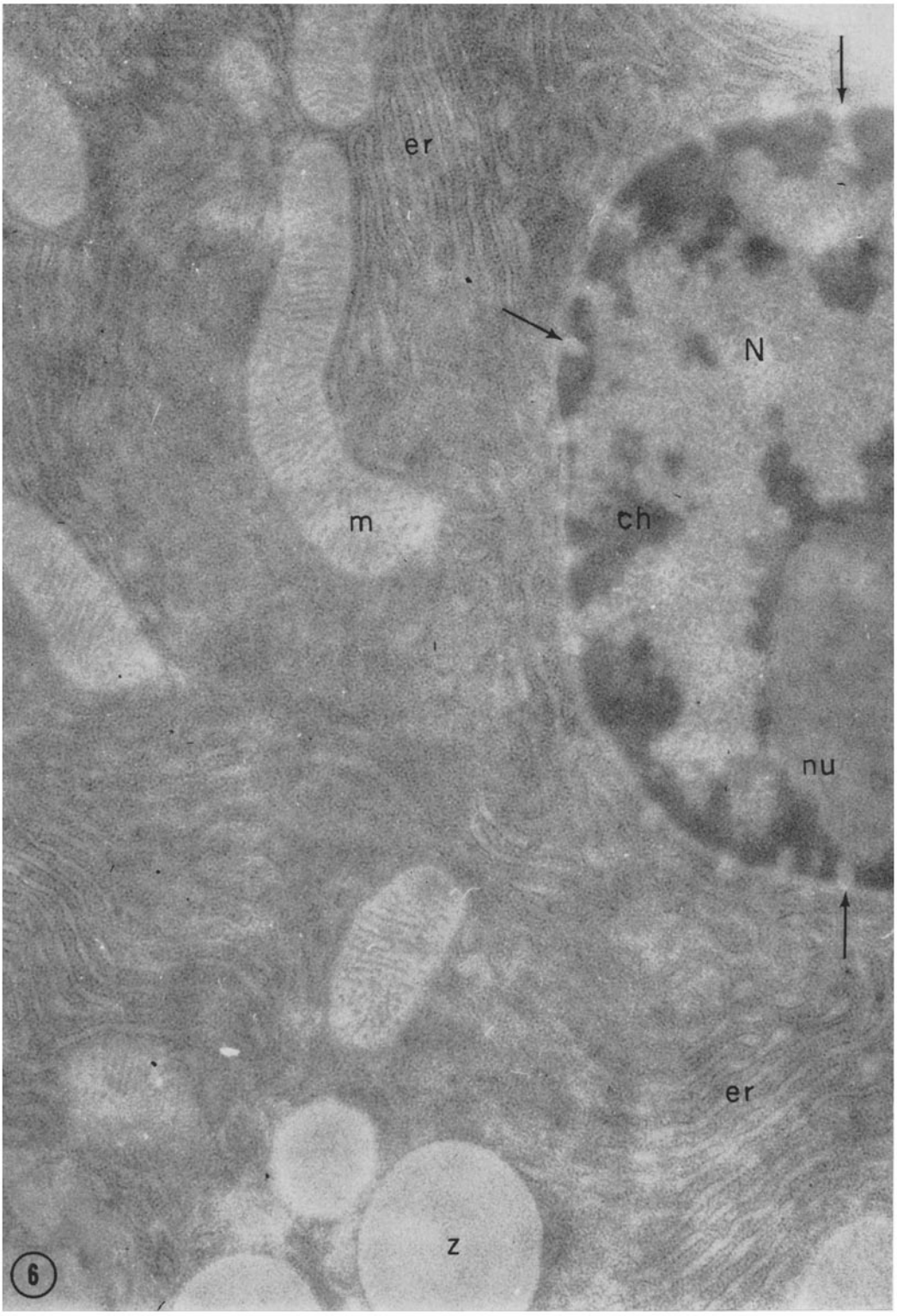

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was always found directly applied to the nucleolus, forming an incomplete capsule in which the intervening spaces provided direct contact with the karyoplasm (Fig. 8). As in the other parts of the nucleus, some circular and longitudinal profiles were observed in the nucleolus, as well as occasional solid granules.

tion, other channels appeared to end internally in the chromatin, or more commonly in the karyoplasm. The clear spaces that marked the channels extended beyond the limit of the nuclear pore, frequently reaching the hyaloplasm between the levels of the first and second cisternae of the endoplasmic reticulum (Figs. 6 to 8 and 10). The main

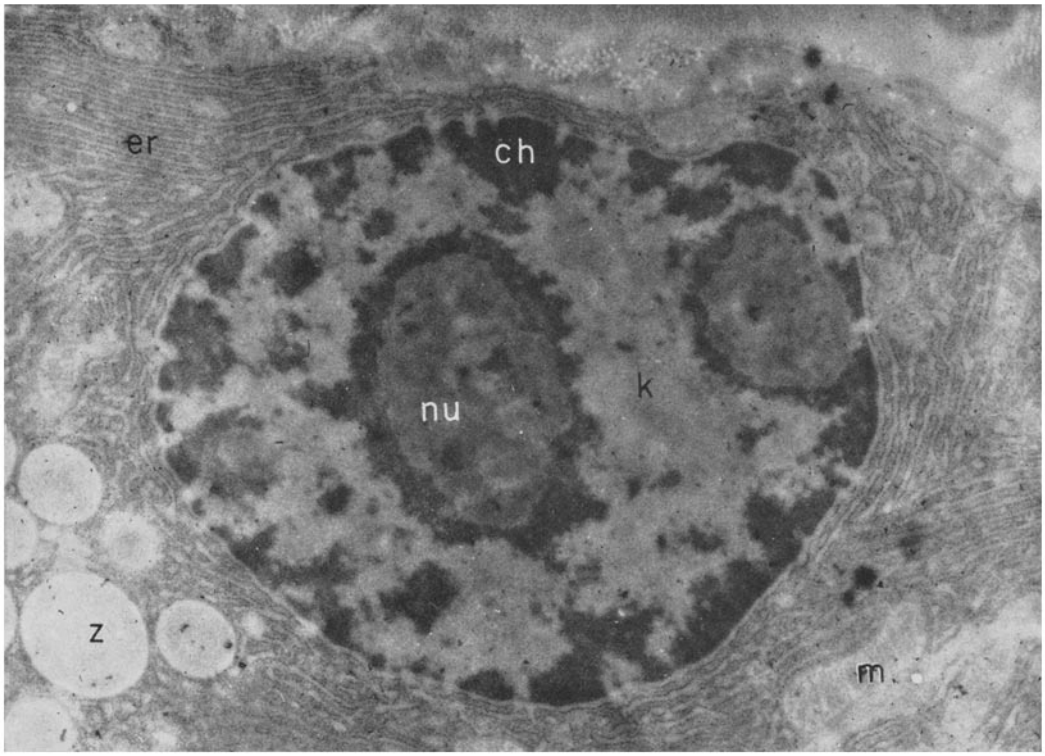

FIGURE 7

General view of a pancreatic acinar cell fixed in glutaraldehyde alone. Nucleus shows over-all detail and clear differences in density between the nucleolus (*nu*), chromatin (*ch*), and karyoplasm (*k*). The plasma membrane (top) is not demonstrable. In the intercellular space, negative images of collagen fibers are observed. Mitochondrion, *m*; endoplasmic reticulum, *er*; zymogen granule, *z*. × 11,000.

No nuclear membranes were discernible, but the nucleocytoplasmic interface was very sharply demarked. On the nuclear side of this limit, chromatin formed an almost continuous layer interrupted by nuclear pores. These pores consisted of relatively clear spaces which were less dense than the karyoplasm and extended like channels on both sides of the nucleocytoplasmic interface. Toward the interior of the nucleus, the channels reached in some instances the part of the contour of a peripheral nucleolus which was not surrounded by chromatin. Depending on the plane of the section, other channels appeared to end internally in the chromatin, or more commonly in the karyoplasm. The clear spaces that marked the channels extended beyond the limit of the nuclear pore, frequently reaching the hyaloplasm between the levels of the first and second cisternae of the endoplasmic reticulum (Figs. 6 to 8 and 10). The main features of the above description of the nuclear detail, such as the distribution of chromatin and presence of pores, are in agreement with those of Swift (47) and Watson and Aldridge (48).

In the cytoplasm of the pancreatic acinar cells, the most striking finding with the glutaraldehyde-fixed material was the excellent preservation of the endoplasmic reticulum (Figs. 6 and 7), which appeared as a parallel array of cisternae with a clear content and a rough surface. However, at higher magnification (Fig. 9) it was clear that the limits of the cisternae were being visualized

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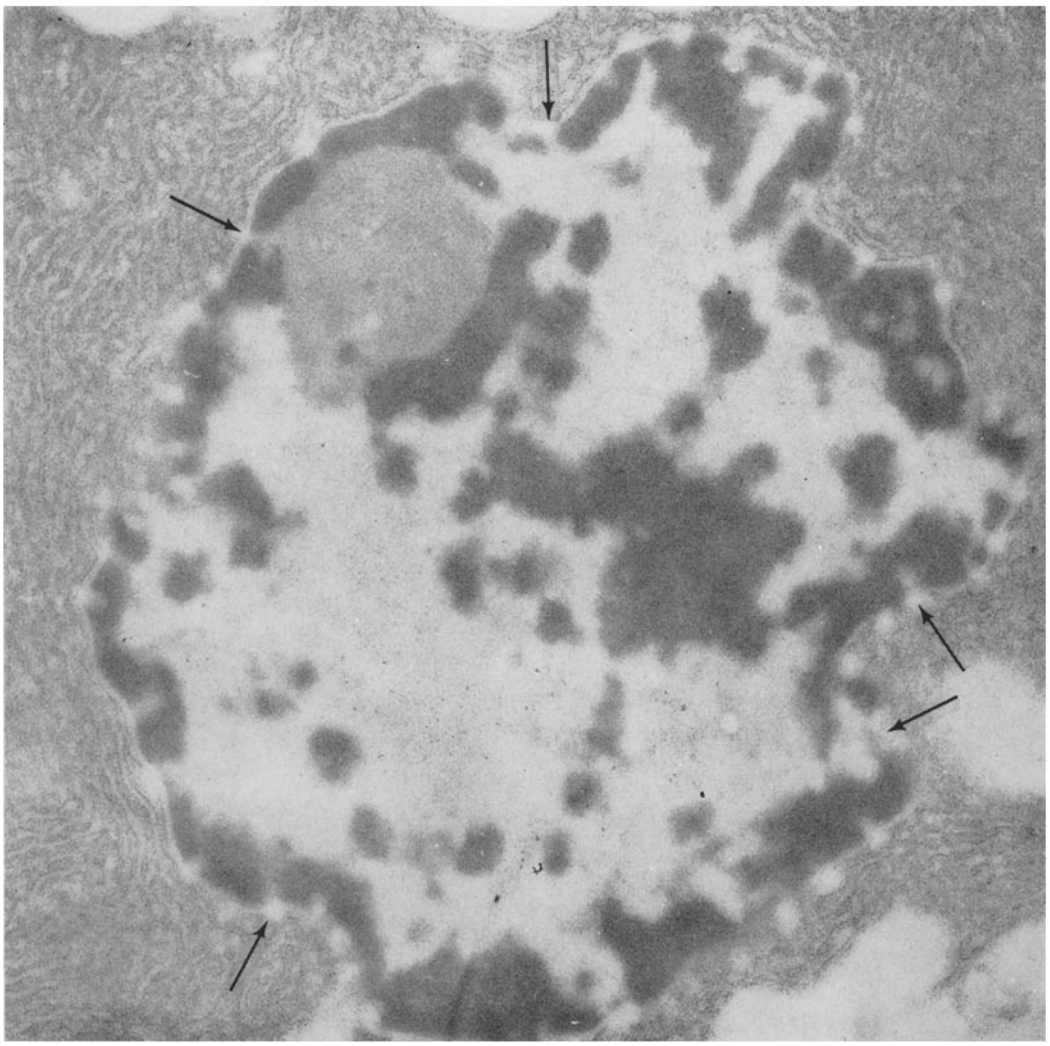

FIGURE 8

Nucleus of pancreatic acinar cell fixed in acrolein, showing structure similar to that of nuclei fixed in glutaraldehyde (Figs. 6 and 7). Nuclear pores are indicated by arrows. × 26,500.

only by the presence of the ribonucleoprotein particles, ordered linearly, and the membranous component was lacking. The hyaloplasm between the cisternae was relatively dense and contained some particles similar to those limiting the cisternae.

Mitochondria were also clearly visible. Although the outer membranes of mitochondria were not discernible, the cristae appeared as parallel double lines, denser than the intervening matrix (Figs. 6 and 10). Dense mitochondrial granules, commonly visualized in the matrix in osmium tetroxide–fixed preparations, were not apparent in material fixed in the aldehydes alone.

In the vicinity of the nucleus, a Golgi apparatus could be recognized by the presence of a system of vacuoles and dilated sacs with a clear content (Fig. 11). As was the case with the rough endoplasmic reticulum, membranes were not apparent, and the lack of ribonucleoprotein particles accounted for a more indefinite limit of these structures. They were visible mainly because of the difference in

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density between the content of the vesicles and the surrounding substance of the hyaloplasm.

Zymogen granules appeared as round or oval homogeneous bodies of low density (Figs. 6, 7, and 11). On the other hand, dense bodies (lysosomes) appeared as round structures of greater density than the zymogen granules. In other tissues (*e.g.*, liver or kidney) these bodies were the densest cytoplasmic components found. No limiting membrane of these bodies was observable, and the internal dense material was homogeneous and did not show the curled membranes and the granulants commonly seen in osmium tetroxide–fixed material. Lipid droplets were dissolved by the embedding procedure, leaving clear spaces. At the cell surface a plasma membrane was lacking, but the cell limit was recognizable because of the difference in density between the peripheral hyaloplasm and the less dense intercellular substance. Collagen fibers in the intercellular spaces appeared as negative images immersed in a homogeneous ground substance (Fig. 7).

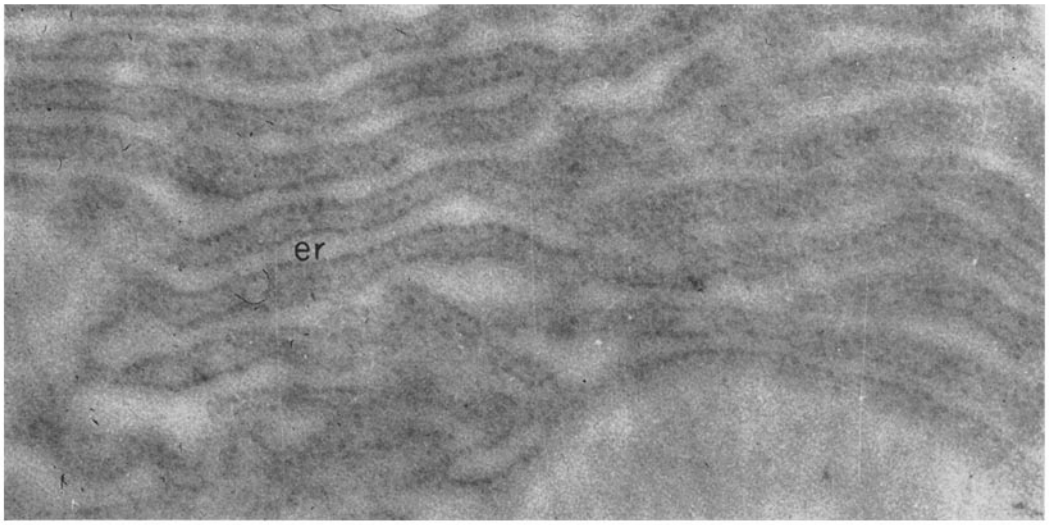

FIGURE 9

Higher magnification of the endoplasmic reticulum (*er*) of a pancreatic acinar cell fixed in glutaraldehyde. The membranes of the parallel cisternae are not visible but the ribosomes delineate their location. A part of a mitochondrion is seen at the bottom. × 65,000.

MATERIAL POSTFIXED IN OSMIUM TETROXIDE

When tissue already fixed in one of the aldehydes was refixed in osmium tetroxide, a quality of preservation comparable, and in some cases (*e.g.* glutaraldehyde and acrolein) equivalent, to that obtained with osmium tetroxide alone was achieved. For adequate postfixation in osmium tetroxide of tissues fixed in the aldehydes, it was necessary to introduce an intermediary step in which the blocks were washed in buffered sucrose solution several hours to overnight (minimum time depending on the block thickness). The introduction of this step resulted from the observation that when aldehyde-fixed blocks were immediately postfixed in osmium tetroxide, the contrast obtained in the final image was low, and usually cytoplasmic membranes were not apparent or prominent (Fig. 12) even if the postfixation was prolonged (4 hours), or the usual concentration of the osmium tetroxide doubled (2 per cent). The requirement of washing before postfixation was less important in the cases of tissues fixed in hydroxyadipaldehyde, pyruvic aldehyde, acetaldehyde, crotonaldehyde, and methacrolein. As could be expected, those fixatives which gave excellent preservation when used alone also gave excellent results after postfixation in osmium tetroxide. In addition, the quality of the preservation of the tissues fixed in the other aldehydes appeared considerably improved after similar postfixation. We interpret this to mean that the poorer results ob-

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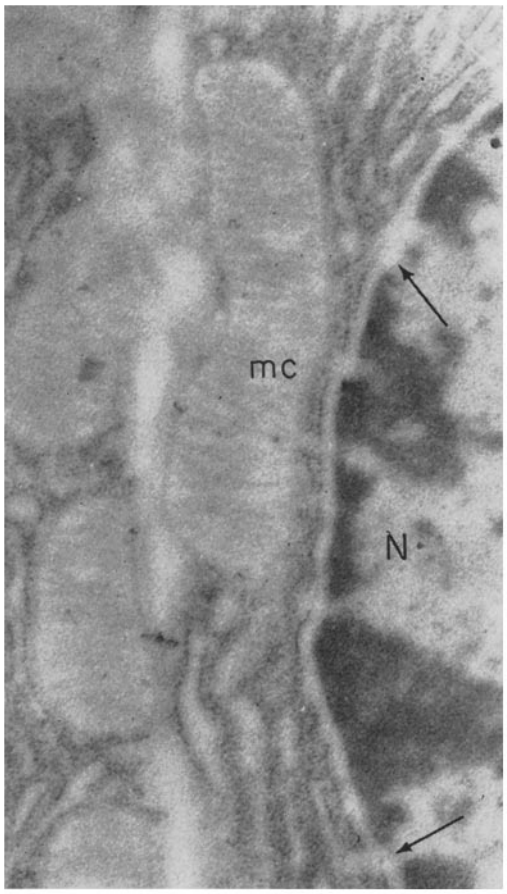

FIGURE 10

Parts of two pancreatic acinar cells fixed in glutaraldehyde, showing several mitochondria with visible cristae (*mc*). Outer mitochondrial, plasma, and nuclear membranes are not apparent. The edge of a nucleus (*N*) shows several pores (arrows) between the chromatin masses, and the pore at the bottom appears to extend beyond the limit of the first cisternae of the endoplasmic reticulum. × 31,000.

tained with some of these fixatives alone (pyruvic aldehyde, acetaldehyde, and hydroxyadipaldehyde) could be attributed at least partly to damage to the specimen (extraction and disruption) produced by the dehydration and embedding procedures.

Postfixation in osmium tetroxide in these cases therefore appeared necessary not only to add contrast and develop membranous sharpness, but to stabilize the fine structure already maintained by the aldehydes so that it could withstand Epon embedding. The maintenance of structure by the aldehydes was emphasized by the fact that fixed blocks could be stored for several months and produced excellent morphological results after the postfixation in osmium tetroxide (Fig. 13). In spite of the general improvement by postfixation of tissues fixed in any of the aldehydes, they still could be classified into the original groups as to the quality of preservation.

The size of the block to be fixed in an aldehyde was not so much a critical factor as it is in the case of osmium tetroxide; in fact, relatively large blocks could be fixed and stored in cold buffer for long periods, at the end of which smaller blocks could be trimmed from the large ones for postfixation in osmium tetroxide, without loss of quality. This indicated that the aldehydes, at the concentrations and for the periods of fixation used, penetrated enough to complete the fixation of blocks no larger than 1 cm × 1 cm × 0.4 cm without noticeable postmortem alterative changes.

It was found that, while material fixed in the aldehydes alone gave satisfactory results after methacrylate or Epon embedding, material that was postfixed in osmium tetroxide could only be embedded in Epon. Sections of methacrylate-embedded tissue that had been doubly fixed were always seriously damaged by the electron beam and showed a degree of sublimation far in excess of that found in tissues fixed in osmium tetroxide alone. It should be noted that the contrast of the tissues fixed in aldehydes, washed and postfixed in osmium tetroxide, and embedded in Epon appeared greater than could be expected of tissues fixed in osmium tetroxide only, and embedded in Epon. This allowed direct observation of the former tissues without staining with heavy metal techniques (Figs. 13 and 14).

As mentioned before, the most pronounced morphological finding in the tissues postfixed in osmium tetroxide was the appearance of cellular membranes. This together with an increase in contrast produced an image largely indistinguishable from that obtained with osmium tetroxide alone (Figs. 13 and 14). The membranes that now appeared as thin, dense lines, approximately 75 A in width, included plasma, nuclear, and mitochondrial membranes and those of the granular and agranular reticulum (Figs. 13, 14, and 18 to 21). In some cases (methacrolein and pyruvic aldehyde) mitochondrial membranes were demonstrated while the other cell membranes still lacked

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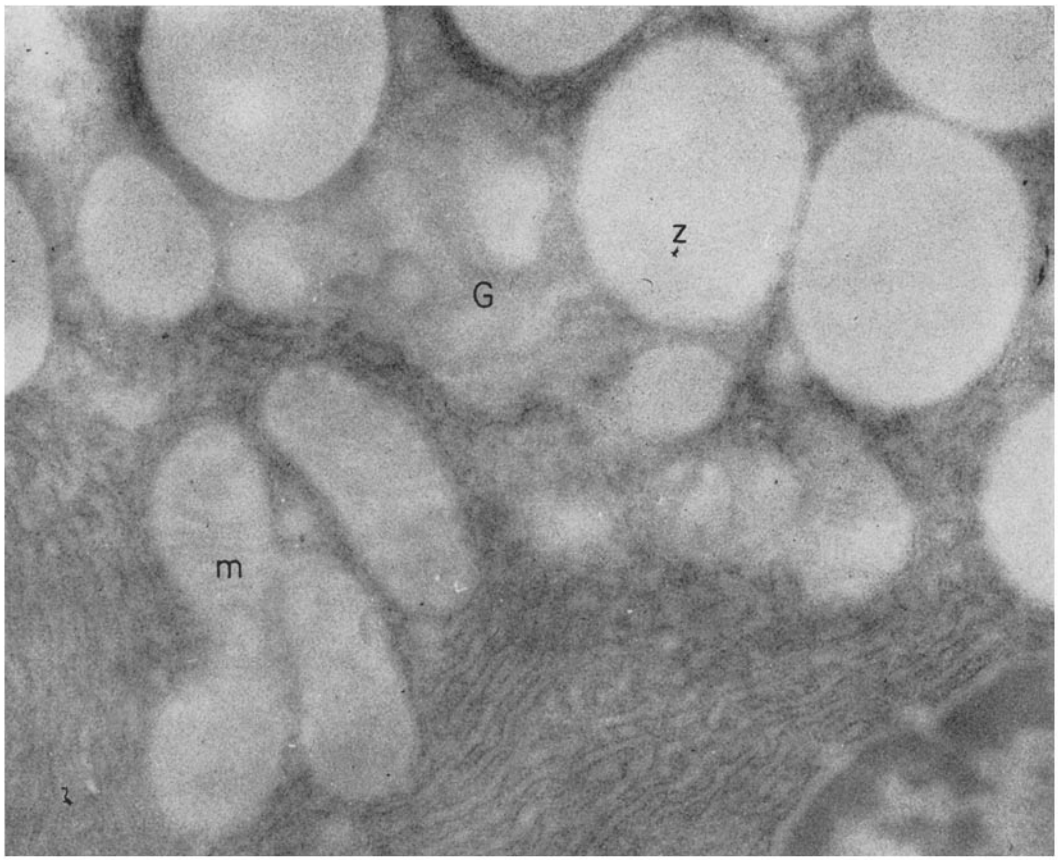

FIGURE 11

Golgi region (*G*) of a pancreatic acinar cell fixed in methacrolein. Membranes of Golgi cisternae and vesicles are lacking, as well as those enveloping the mitochondria (*m*) and zymogen granules (*z*). Note that the cytological details with this fixative do not differ appreciably from those shown with previous fixatives. × 31,000.

density (Figs. 15 to 17). Additional details after refixation included the presence of dense, round granules in the mitochondrial matrix (Figs. 15 and 16), the retention of lipid droplets, an increase of the cytoplasmic dense bodies that showed a single limiting membrane, and a greater density of the ribonucleoprotein particles (Fig. 14).

The mitochondrial matrix, the ground substance of the hyaloplasm, and the structureless contents of the endoplasmic reticulum, as well as

FIGURE 12

Micrograph of pancreatic acinar cell fixed in glutaraldehyde and refixed in 1 per cent osmium tetroxide for 2 hours without intermediate washing. Though the general contrast has increased greatly, nuclear and cytoplasmic membranes are still not visible. The nuclear channels (arrows) are still recognizable on the nuclear side but do not extend through the pores into the cytoplasm. Chromatin (*ch*) is somewhat dispersed and numerous particles occupy the karyoplasm. Density of the nucleolus (*nu*), the chromatin masses, zymogen granules (*z*), and mitochondria (*m*) has increased considerably. Collagen fibers (*c*) appear as positive images in the intercellular space. × 15,000.

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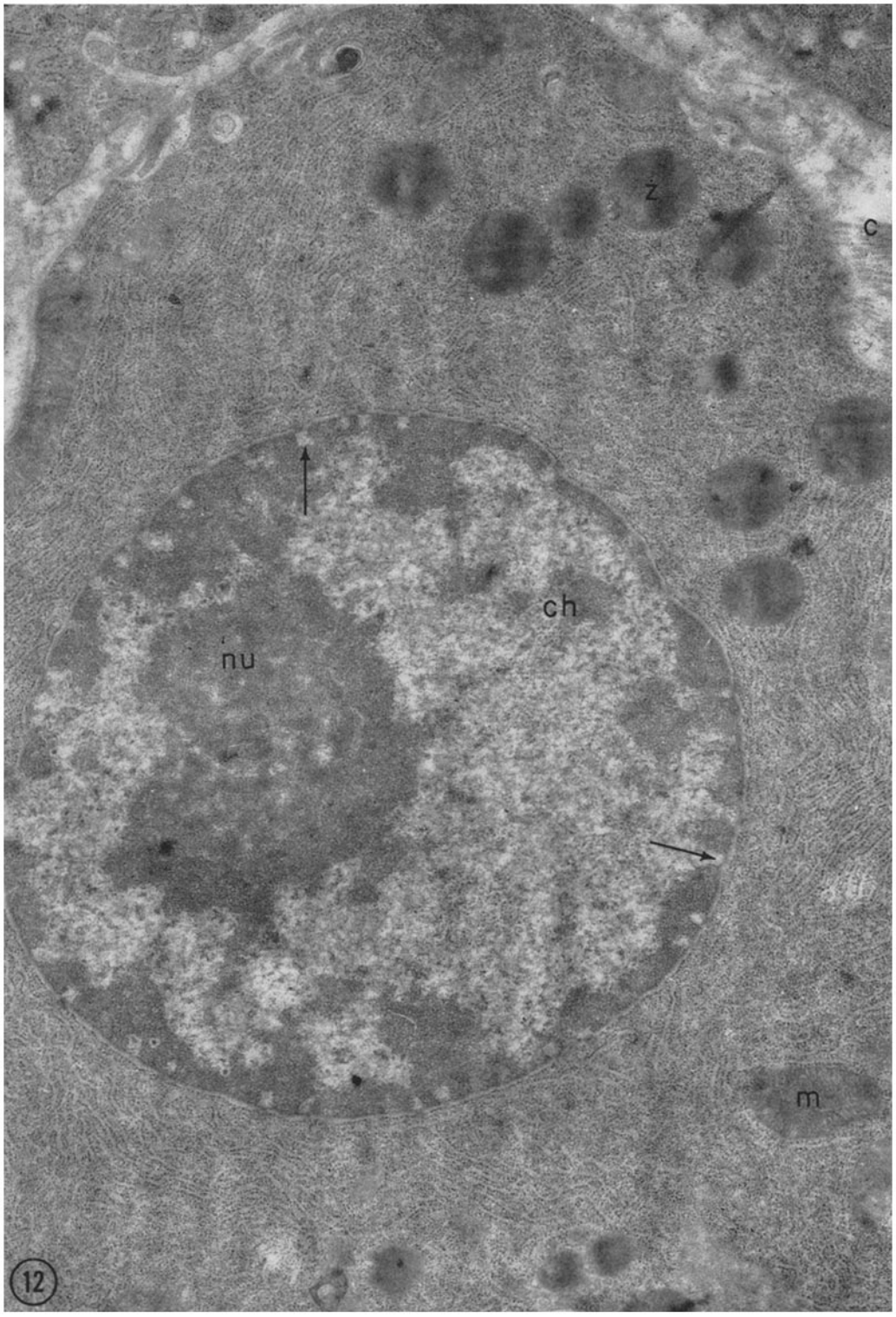

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the contents of intercellular and pericapillary spaces, appeared denser after the double fixation than after osmium tetroxide alone. This suggested that in those places a greater proportion of material is retained by the use of the former procedure. This suggestion was further borne out by the observations on rat liver glycogen areas.

It was found that material prefixed in glutaraldehyde or acrolein (but not in glyoxal, crotonaldehyde, or hydroxyadipaldehyde) and refixed in osmium tetroxide contained numerous dense particles of diffuse contours (about 300 A in diameter) occupying the glycogen areas (Fig. 18). It should be recalled that these particles in rat liver are not ordinarily demonstrated as conspicuously by osmium tetroxide fixation alone without lead staining (49). The particles appearing when osmium tetroxide was used after glutaraldehyde or acrolein prefixation were similar to those appearing in rat liver after potassium permanganate (5) fixation or in other rat tissues or the livers of other species after osmium tetroxide fixation. In the case of the material prefixed in acrolein, the particles were grouped to form rosettes of 10 to 12 units or disposed in linear and frequently branched arrays. In material prefixed in glutaraldehyde the particles were more irregular, and sometimes contained hollow centers (Fig. 18). In material prefixed in the other aldehydes, the glycogen areas either were structureless spaces or were filled with a homogeneous material of very low density. Particles of greater density and size than these found in the glycogen areas were encountered occupying large vesicles, forming units with the same characteristics as those described in association with the Golgi region (50) (Fig. 19).

Lipid droplets in muscle (Fig. 13), liver, pancreas, and adrenal (Fig. 20) treated by double fixation in aldehyde and osmium tetroxide were retained through the embedding except occasionally when acrolein was used as the first fixative. Since lipid droplets were almost always lost when the aldehyde-fixed material was embedded without postfixation, it appears that the lipids are retained by the aldehydes and further rendered insoluble for the processes of dehydration and embedding by the osmium tetroxide.

Changes in the nucleus were obvious after the double fixation when the structure was compared with that found after aldehyde fixation alone (Figs. 20 and 21). In general, the nucleus appeared more like that fixed in osmium tetroxide. The double membrane surrounding the nucleus was interrupted by occasional pores, but the clear spaces that marked the channels and extended through the pores in the aldehyde-fixed material were absent after postfixation in osmium tetroxide. Chromatin material appeared dispersed (and/or dissolved), since the masses at the periphery of the nucleus and around the nucleolus were smaller and fewer. The dense particles of the chromatin appeared increased in density and many of them were found free or clumped in the karyoplasm (Figs. 20 and 21). This arrangement of dense particles, absent in the material fixed in aldehyde alone, could be accounted for by either a redistribution of the chromatin material during postfixation or an increase in density of some of the karyoplasmic material. Nucleoli increased in density as a result of postfixation in osmium tetroxide and appeared to be composed of finely granular dense material (Fig. 20). Although a few annular structures could occasionally be discerned in chromatin masses after double fixation, they were absent from the karyoplasm.

Collagenous fibers after double fixation presented the usual image with a periodicity of approximately 600 A that is obtained with osmium tetroxide alone (Fig. 12). Basement membranes of homogeneous low density were conspicuous after the double fixation.

When sections of aldehyde-fixed, Epon-embedded material were stained with a solution of 1 per cent osmium tetroxide applied overnight, an increase in density of ribonucleoprotein

FIGURE 13

Micrograph of diaphragmatic muscle fixed in glutaraldehyde for several hours and stored in cold buffer containing sucrose for several months before refixation in 1 per cent osmium tetroxide. Parts of two fibers are depicted. Mitochondrial (*m*) and plasma membranes are conspicuous. Thick and thin myofilaments are visible as well as the bands of the sarcomere. A solitary lipid droplet closely associated with the mitochondria has been preserved throughout the procedures. Dense particles, presumably glycogen, are seen in a part of the sarcoplasm (left). × 42,500.

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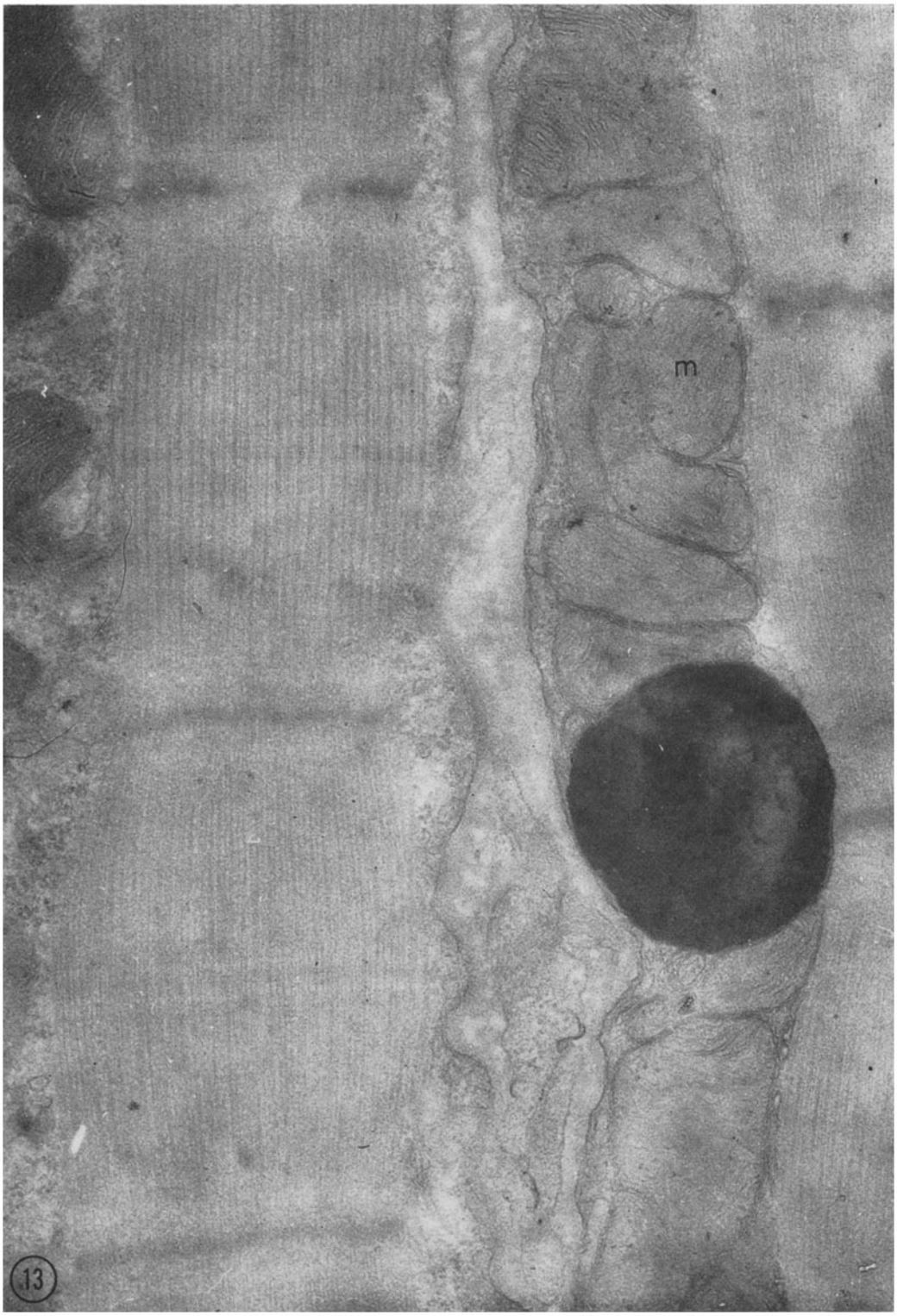

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particles, ground substance of the hyaloplasm, and content of the cisternae was observed. The membranes of the endoplasmic reticulum, nucleus, cell surface, and Golgi apparatus were either lacking or faintly outlined. Mitochondrial membranes and denser granules of the mitochondrial matrix were occasionally developed by this treatment, though in mitochondria the outer membranes were less apparent.

When non-osmicated sections were stained with lead hydroxide, a considerable increase in density of the cytoplasmic ribonucleoprotein particles was observed (Fig. 22). This increase in density caused by lead staining was not paralleled in any other cellular structure except the nucleolus. In this organelle dense granules (approximately 100 A in diameter), not apparent before staining, were clearly visible in a background of augmented density (Figs. 22 and 23). Chromatin masses and other nuclear components, except for occasional granules, were not stained by the lead treatment. In preliminary experiments it has been observed that different staining procedures, *e.g.* potassium permanganate (51), phosphotungstic acid, and uranyl salts (52), can be applied to material fixed in aldehyde alone.

Cytochemical Experiments

Light Microscopy

The results of the preliminary histochemical tests with the light microscope are compiled in Table II and some are illustrated in Figs. 2 to 5. For the present work, we should like to focus on the retention of enzymatic activity in a limited number of tissues fixed in the various aldehydes. A more detailed study of the distribution of activity will be presented later.

The most interesting finding concerned the demonstration of succinic dehydrogenase and cytochrome oxidase activity (Fig. 5) of heart muscle in hydroxyadipaldehyde-fixed tissue. After fixation for 6 hours, these activities were considerable and clearly limited to mitochondria, and they were still demonstrable, though greatly diminished, after 24 hours' fixation. In addition, moderate activity of these enzymes was also found after fixation in glyoxal for 2 hours, and a trace of succinic dehydrogenase activity remained after fixation for the same length of time in crotonaldehyde and pyruvic aldehyde. Weak cytochrome oxidase activity was also present after fixation for 2 hours in acetaldehyde, pyruvic aldehyde, or formalin. In the succinic dehydrogenase experiments, no endogenous dehydrogenase activity was noted, since omission of succinate resulted in a negative reaction. Addition of malonate to the regular incubating medium for succinic dehydrogenase activity also inhibited the reaction. The extreme periphery of the blocks usually showed no reaction regardless of the kind of fixation. With hydroxyadipaldehyde-fixed tissues the cytochrome oxidase reaction was intensely positive after 5 minutes of incubation, whereas with the other fixatives the incubation time was prolonged from 30 to 60 minutes. The most rapid reaction for succinic dehydrogenase activity occurred in 10 minutes.

In the experiments for the demonstration of cytochrome oxidase, addition of catalase did not affect the rate of the reaction or the distribution of the final product. The addition of cytochrome *c* enhanced the reaction in all instances in which a positive reaction was observed without cytochrome *c*. In addition, in glutaraldehyde- and methacrolein-fixed muscle, a moderate reaction appeared when cytochrome *c* was added, whereas no reaction occurred without the supplementation of the medium with this substrate. In these instances fixation obviously destroyed or removed the activity of cytochrome *c* but did not suppress completely that of the cytochrome oxidase.

In the experiments for the localization of DPNH or TPNH diaphorase activities in heart muscle, intensely positive results occurred with five of the fixatives and moderate results with two (Table II). Only acrolein- and pyruvic aldehyde-fixed material gave negative results. The reaction was complete in from 10 to 20 minutes, and in all

FIGURE 14

Fibroblast in the endoneurium of rat sciatic nerve fixed in glutaraldehyde and stored for 1 month before refixation in osmium tetroxide. The increased contrast of this material as well as of all other doubly fixed cells is due solely to fixation. Free and attached ribosomes are very conspicuous. Within the cisternae of the endoplasmic reticulum a relatively dense material has been retained. × 35,000.

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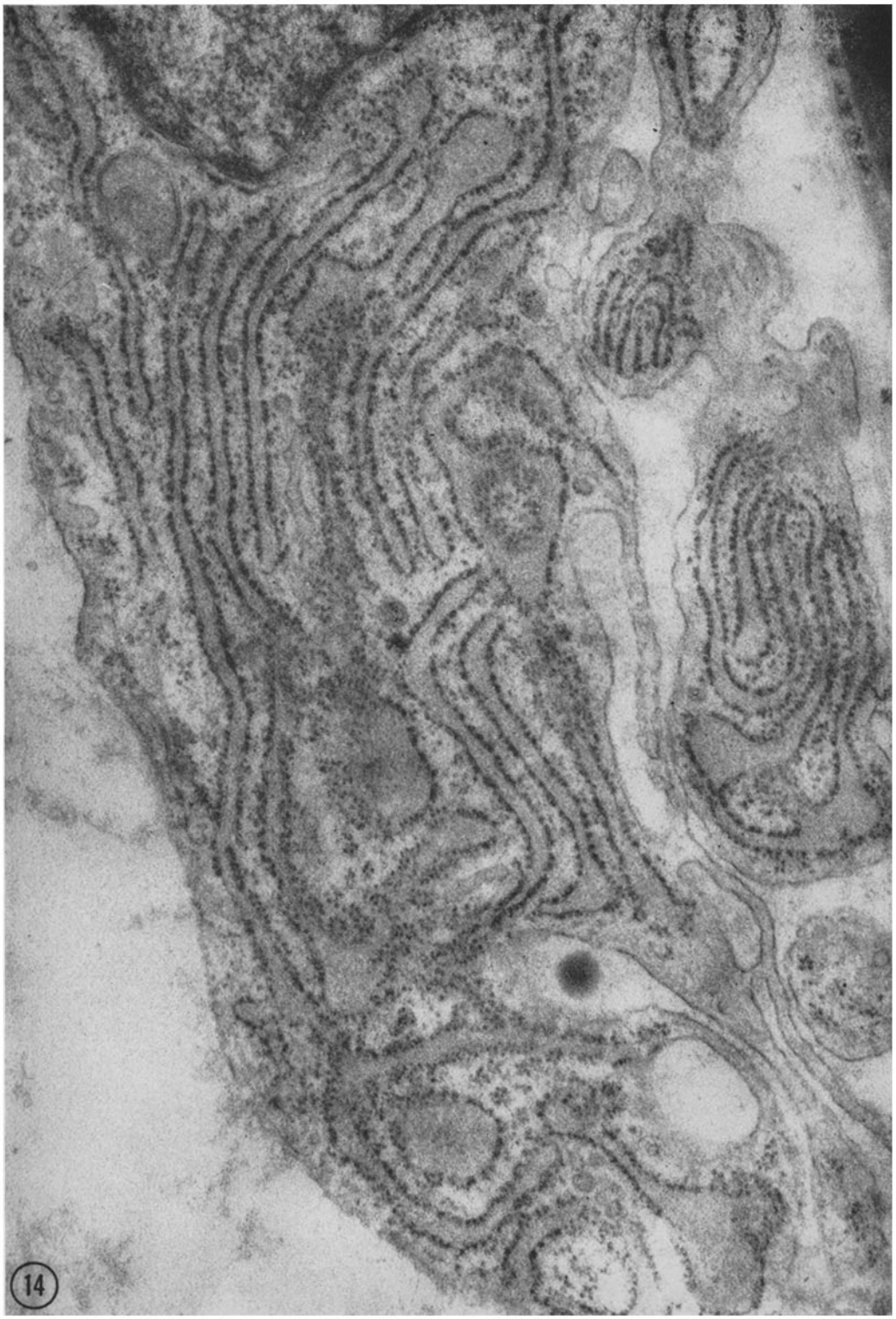

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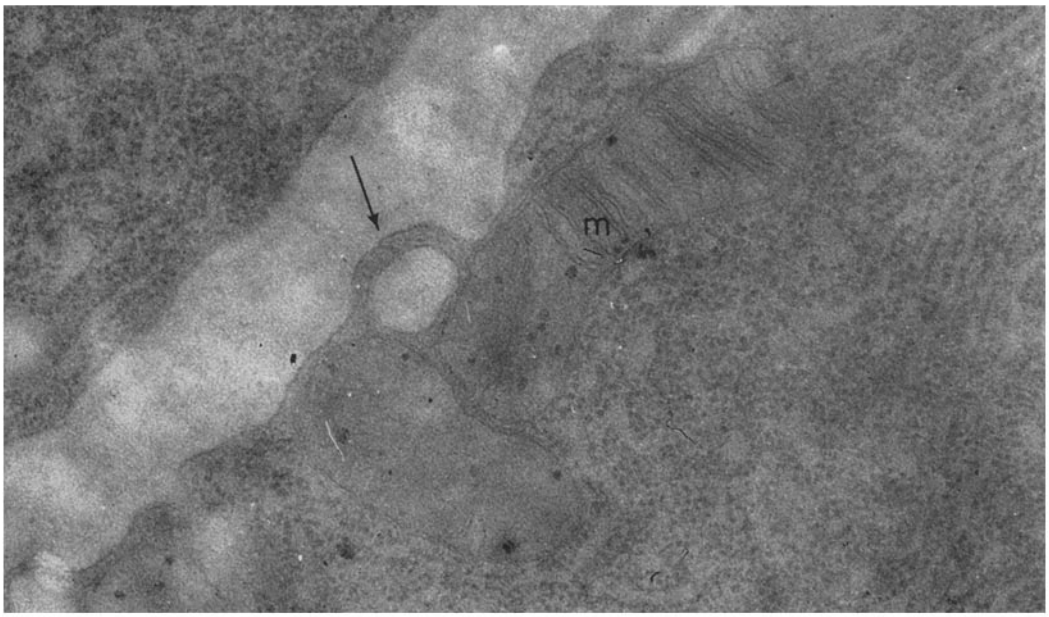

FIGURE 15

After fixation in methacrolein and refixation in osmium tetroxide, mitochondrial (*m*) and plasma membranes (arrow) of pancreatic acinar cells are visible. Several dense granules may be noted in the mitochondrial matrix. × 40,500.

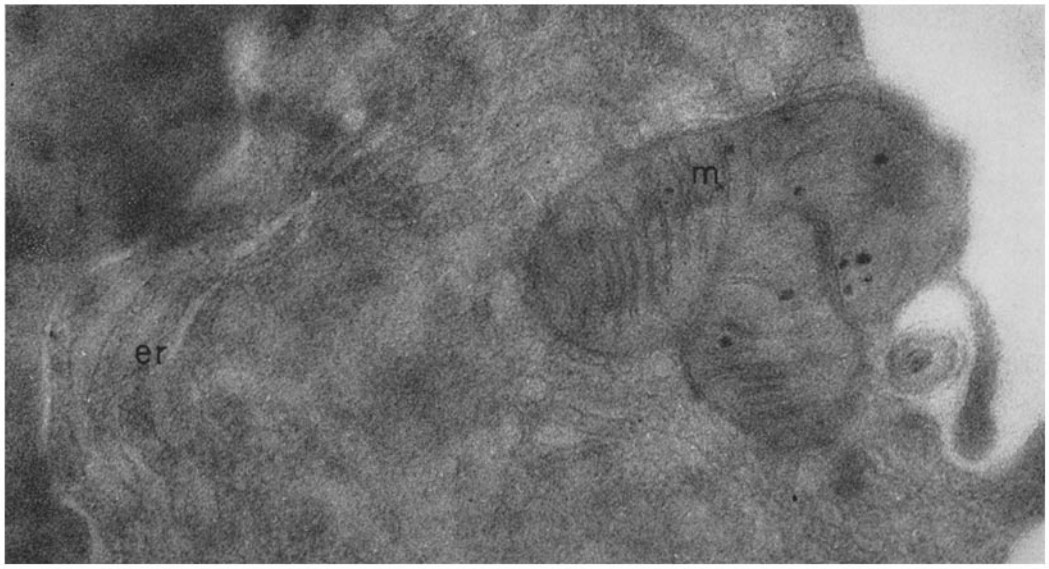

FIGURE 16

Refixation in osmium tetroxide of material (rat pancreas) fixed in pyruvic aldehyde develops mitochondrial (*m*) membranes and dense mitochondrial granules. Though this treatment enhances the general contrast, it frequently fails to show nuclear and plasma membranes as well as those of the endoplasmic reticulum (*er*). × 49,000.

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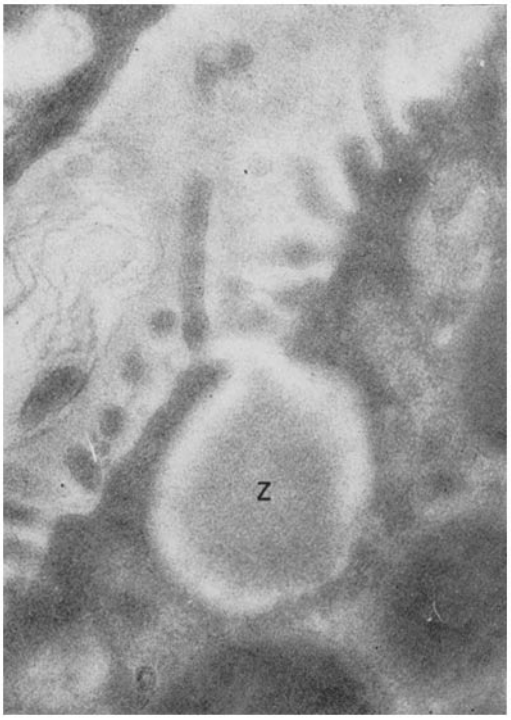

FIGURE 17

Periphery of pancreatic acinar cell fixed in acetaldehyde and refixed in osmium tetroxide, showing the opening of a zymogen granule (*z*) at the cell border. Cell membranes are poorly developed after this procedure. × 35,000.

cases intense mitochondrial, as well as a more faint sarcoplasmic, reaction occurred. Omission of the substrate resulted in a negative reaction.

Carboxylic acid esterase activity in the cytoplasm of liver and kidney cells demonstrable with either naphthyl acetate or thiolacetic acid as substrate was negative with pyruvic aldehyde and positive to a varying extent with the other fixatives. In the cases of the material fixed in glutaraldehyde (Fig. 4), formalin, or acrolein, the staining of cytoplasmic bodies (lysosomes), especially by the thiolacetic acid method, was conspicuous, but it was not apparent in the other cases. This reaction was presumably due to the activity of cathepsin C, which, unlike the esterase activity, was not inhibited by E 600.

With the same substrate, discrete staining of the myoneural junctions occurred rapidly in glutaraldehyde- and formalin-fixed material, was somewhat delayed after glyoxal fixation, and was absent after acrolein. The final product appeared restricted to the synaptic gutters, and this reaction was completely inhibited by eserine (10^{-4} M) or DFP (10^{-4} M).

Acid phosphatase activity localized in dense cytoplasmic bodies (lysosomes) of renal tubular and hepatic cells was demonstrated after 30 minutes' to 1 hour's incubation with all fixatives (5-hour fixation) except acrolein (1-hour fixation) and glyoxal, which were negative. After 30 minutes' fixation in acrolein, a moderately positive result was obtained. Although excellent localization was obtained by the lead salt method in glutaraldehyde, formalin, methacrolein, crotonaldehyde, and acrolein (30 minutes), nuclear staining with the same batch of incubating medium was found in the tissue fixed in pyruvic aldehyde, acetaldehyde, and hydroxyadipaldehyde. More reliable results (no nuclear staining) with the same fixatives were obtained when the azo dye technique was used, in which the coupling was performed with hexazonium salt of pararosaniline. Both tartrate and sodium fluoride completely inhibited the activity of this enzyme. Alkaline phosphatase activity localized to the brush borders of the proximal tubules of the kidney was intense with all fixatives except acrolein, which showed a slight reaction.

Hepatic enzymes that hydrolyze ATP were absent only after acrolein fixation. The surface of the hepatic cells around the bile canaliculi reacted moderately after glutaraldehyde and strongly after formalin, glyoxal, methacrolein, crotonaldehyde (Fig. 2), acetaldehyde, and hydroxyadipaldehyde. In the latter two cases, a granular staining (mitochondrial?) also occurred. This cytoplasmic staining was very conspicuous after pyruvic aldehyde fixation, in which no biliary canaliculi were reactive.

A variable reaction in liver cells occurred when AMP was used as a substrate for 5-nucleotidase. While the reaction was negative in tissues fixed in acrolein, in the other fixed material a positive reaction took place that varied in its localization. In glutaraldehyde-, crotonaldehyde-, and hydroxyadipaldehyde-fixed material, discrete intensely reactive areas occurred at the periphery of hepatic cells. With the other fixatives, a variable cytoplasmic reaction ensued.

When glucose-6-phosphate was used as substrate, only hydroxyadipaldehyde-fixed liver showed a positive cytoplasmic reaction (Fig. 3). This is the only fixative that has been found to

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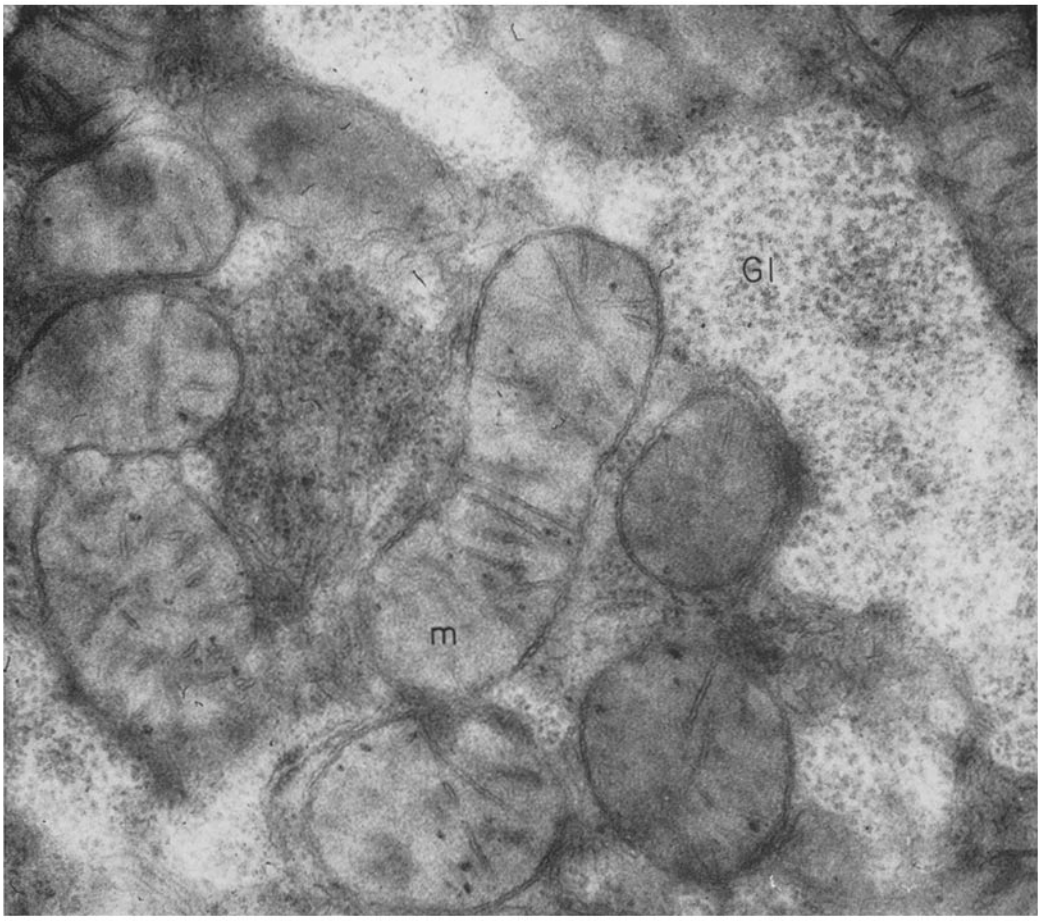

FIGURE 18

Mitochondria and glycogen areas of a rat hepatic cell fixed in glutaraldehyde and refixed in osmium tetroxide after several weeks of storage. Note that dense particles of various shapes and sizes occupy the glycogen areas (*Gl*). Mitochondrial (*m*) membranes and dense granules are clearly defined. × 50,000.

date that does not inhibit the activity of this special phosphatase.

ELECTRON MICROSCOPY

We chose for analysis with the electron microscope aldehyde-fixed tissues that showed an acceptable preservation of morphological detail, and to these materials were applied only those methods which at the present stage of development give sharp localization at the submicroscopic levels. Although the preservation of fine structure was less adequate in frozen sections than in small blocks, it should be pointed out that in general the fine structural detail of the fixed tissue was not appreciably altered by the histochemical incubation procedures. Thus, concessions were not made (*e.g.*, avoiding the wash in acetic acid in the acid phosphatase technique) in the methods in favor of preserving morphological detail. This allowed us to obtain a better localization of enzyme activity than would have been the case if we had been forced to modify the technique to fit more stringent requirements in regard to preservation of morphology. This fact points out the primary advantage of aldehyde-fixed tissues over fresh tissues for the combination of histochemistry and electron microscopy.

The product of succinic dehydrogenase activity was demonstrated as moderately dense diformazan deposits either inside or on the surface of mito-

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FIGURE 19

Same material as in Fig. 18. Mitochondria (*m*), and membranes and ribosomes of the endoplasmic reticulum (*er*) are seen. Dense granules occupy vacuoles and sacs of the Golgi system (*G*). × 30,000.

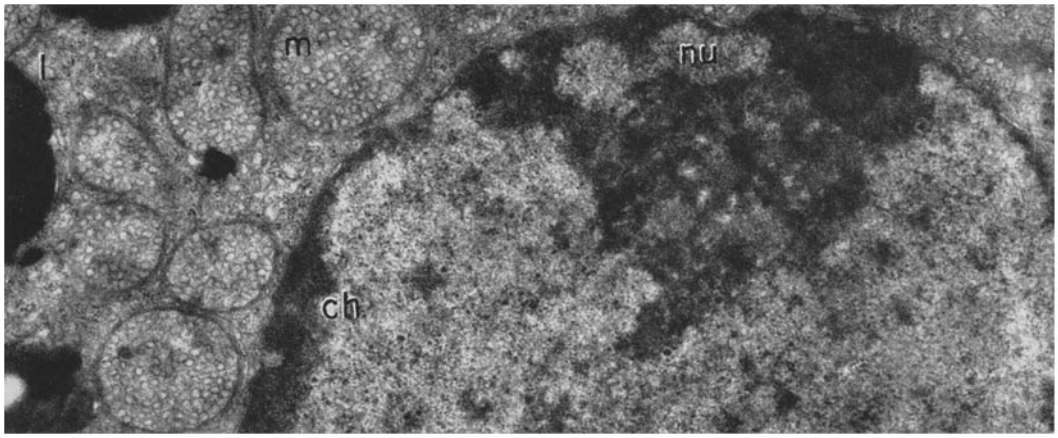

FIGURE 20

Part of a cell from the zona fasciculata of a rat adrenal fixed in glutaraldehyde and refixed in osmium tetroxide after several days of storage. Lipid droplets (*l*) and mitochondria (*m*) with the characteristic vesicular structure are visible in the cytoplasm. The nucleus shows dispersed dense material in the karyoplasm. Peripheral and perinucleolar chromatin masses (*ch*) are fewer and smaller than in the non-osmicated material (Figs. 6 to 8). The nucleolus (*nu*) is denser. × 25,000.

chondria (Fig. 24). Some of the deposits tended to be round, and varied in size from several hundred A to several thousand A. Dense linear deposits were more common, and these occurred in the interior of the mitochondria. The disposition of the latter deposits had the same orientation as the cristae and in many instances appeared to overlie these structures or to fill the spaces between them. In some mitochondria the linear deposits were sparse and well separated.

In others a considerable area of the interior of the mitochondrion was occupied by accumulated adjacent dense parallel linear deposits. Only in some instances was the deposition of the product completely restricted to individual cristae membranes. Sections of the control material (malonate inhibition or substrate lacking) were completely devoid of the product.

deposits was greater in the material incubated in the presence of cytochrome *c*, the distribution was the same. These results were not modified in the material incubated in the presence of catalase, but sodium azide or KCN completely suppressed the reaction.

Rat diaphragm (Fig. 26), liver, and kidney (Figs. 27 and 28) and the visual cells of a toad

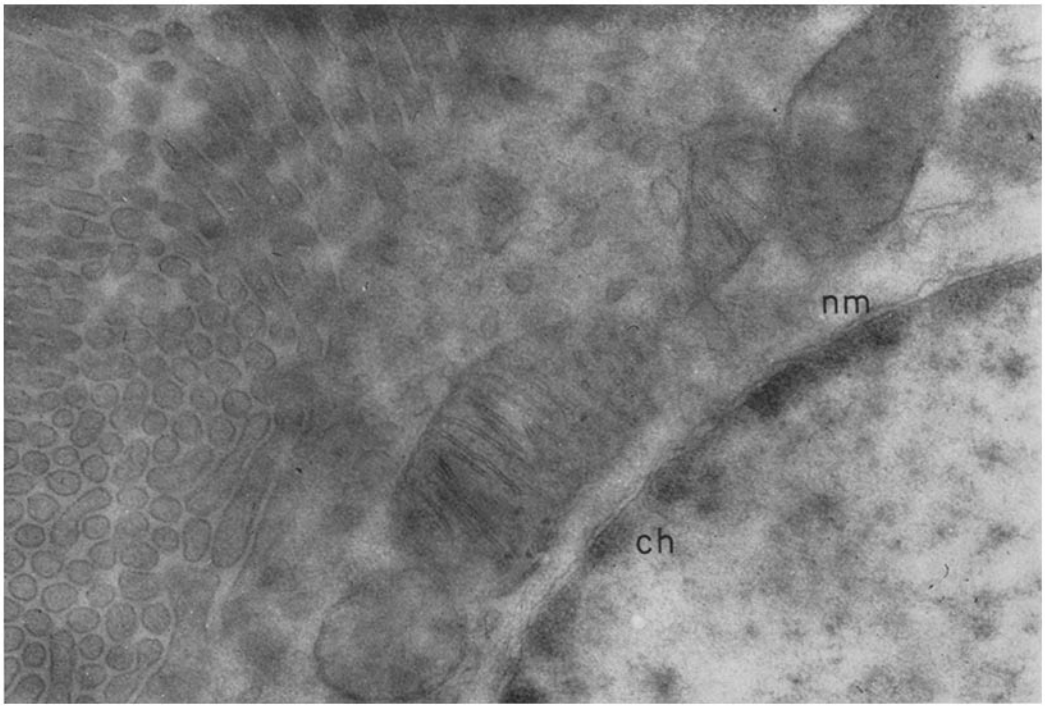

FIGURE 21

Part of a tubular cell of a rat kidney fixed in glyoxal and refined in osmium tetroxide. Cytological details are similar to those encountered with glutaraldehyde after the same procedure. Note the nuclear membrane (*nm*), the absence of clear channels, and the scarcity of peripheral chromatin masses (*ch*). × 30,000.

The localization of the product of cytochrome oxidase activity (Fig. 25) corresponded closely to that of succinic dehydrogenase. As in the previous instance, the deposition of final product in the interior of mitochondria was usually linear and related to the membranes of the cristae, lining or overlying them. However, the number of deposits of various forms and sizes that occurred on the surface of the mitochondria was clearly greater in the heart muscle processed for cytochrome oxidase activity than in that processed for succinic dehydrogenase. While the number of dense

retina were analyzed with the electron microscope for the sites showing activity for the hydrolysis of thiolacetic acid in the presence of lead. In all these tissues dense bodies showed a discrete and intense deposition of final product.

In the proximal convoluted tubule cells, the ground substance of the hyaloplasm contained the final product present as granules of various sizes. These deposits are not illustrated here since they may represent a diffusion of the final product from the dense bodies, having been located in their vicinity and present after E 600 treatment. The

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distal tubular cells of the rat kidney showed, in addition to the reaction in the dense bodies, a delicate stippling of the final product and occasionally very dense accumulations occupying the intramembranous space of the complicated basal infoldings of the plasma membranes (Fig. 28). This reaction was much less intense when thick frozen sections were used instead of small blocks, and in all cases was inhibited by E 600. In general, the agranular vacuolar system of the hyaloplasm did not show activity. However, the vesicles in the Golgi zone showed a fine precipitate and some of them were entirely filled with the dense final product (Fig. 27). In the Golgi region, small dense bodies, as well as large ones when present, showed a positive reaction. This histochemical activity for the splitting of thiolacetic acid in the Golgi zone was not limited to tubular cells. Glomerular epithelial cells also showed activity in the well developed lamellar component of the Golgi apparatus (Fig. 29). Here the reaction was expressed as a discrete staining of the agranular membranes of this region as well as a fine precipitate occupying the interior of the flattened sacs. The presence of an enzymatic activity, possibly that of cathepsin C, in the Golgi zone of glomerular epithelial cells may be related to the role that these cells have in the excretion of protein-containing droplets (53). In correspondence with the previous findings, it was observed that reactive dense bodies, especially small ones, were present also in the vicinity of the Golgi zone of the striated muscular cells. Also in this region the vesicular component of the Golgi apparatus showed a fine deposition of final product. Similar findings of positive dense bodies regularly associated with a reactive Golgi zone were observed in the inner segment of the visual cell of the toad retina. When thick frozen sections of liver tissue were incubated with thiolacetic acid

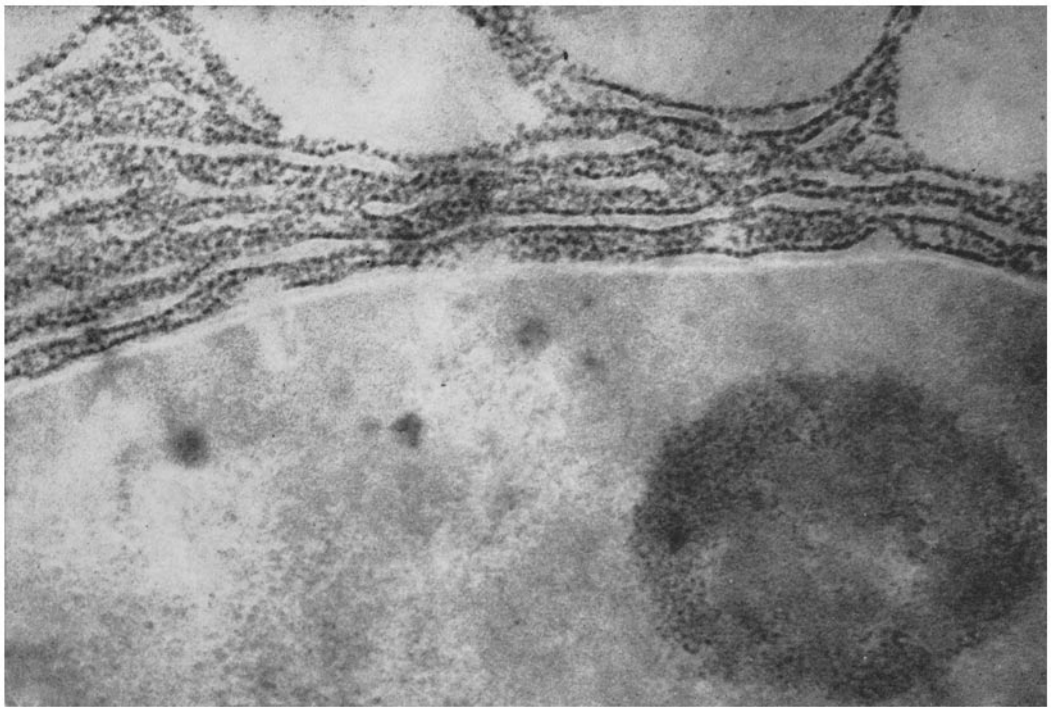

FIGURE 22

Part of nucleus and cytoplasm of a rat pancreatic acinar cell fixed in glutaraldehyde, embedded in Epon, and stained on the section with lead hydroxide. Ribosomes are very dense and conspicuous, as is the nucleolus, within which dense particles are stained. No other cell elements are so intensely stained after this procedure. × 60,000.

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FIGURE 23

Nucleolus of material similar to that shown in Fig. 22. The lead staining shows numerous particles of great density between and surrounding interconnected masses of dense material. Similar structures were not observed with aldehyde or osmium tetroxide fixation alone. × 23,000.

and lead nitrate (Figs. 30 and 31), the final product, in the form of a fine dense precipitate, was encountered in the cisternae of the endoplasmic reticulum of hepatic cells (Fig. 30). This reaction was suppressed by E 600.

Acetylcholinesterase activity of the myoneural junction was also revealed by the thiolacetic acid method. In this instance, the final dense product occurred in glutaraldehyde-fixed material as a fine stippling as well as large clumps in the primary and secondary synaptic clefts. The postsynaptic membrane was distinctly reactive, while the presynaptic membrane showed only patches of reactivity. Synaptic vesicles in the terminal axoplasm also contained very fine granules of final product (Fig. 32). This distribution of final product was similar to that previously found (9), but the activity appeared somewhat inhibited by the glutaraldehyde fixation, and the character of the final product was more punctate.

Acid phosphatase activity in the liver and kidney (Figs. 33 and 34) clearly occurred in many but not all the dense bodies of the epithelial cells. The large dense bodies of macrophages in connective tissue and in Kupffer cells were constantly positive. Although in the parallel sections processed for the light microscope the reaction appeared clearly limited to dense bodies, at the level

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of resolution attained with the electron microscope occasional non-specific deposits of final product were observed. To restrict this drawback, shorter incubations were performed, and, although this resulted in a less intense reaction in dense bodies, less non-specific background reaction occurred.

When ATP was used as substrate with liver fixed in glyoxal, crotonaldehyde, or glutaraldehyde, the reaction appeared on the surface of the microvilli of the bile canaliculi (Fig. 35). In overincubated tissues the entire bile canaliculus was filled with a homogeneous mass of final product. In the incubations that were interrupted earlier, the final product was deposited at the surface of and between some of the microvilli. However, with all fixatives, the sinusoid surface of the hepatic cells also showed some very finely granular final product that was rather diffuse in its localization. In addition, peribiliary dense bodies were also reactive, probably owing to the activity of acid phosphatase. When AMP was incubated for the demonstration of 5-nucleotidase activity in glutaraldehyde-fixed sections, hepatic cell borders were stained, including, though less intensely, the biliary canaliculi (Fig. 36). When IDP was used as substrate (39), the content of the Golgi sacs and vesicles showed strong activity (Fig. 37). These nucleoside di- and monophosphates were also hydrolyzed by the activity in the dense bodies.

Glucose-6-phosphatase activity was retained in hydroxyadipaldehyde-fixed liver, and the product appeared localized to the cytoplasmic areas that contained the rough endoplasmic reticulum (Fig. 38). The final product in this case was a small granular dense deposit associated with or in the vicinity of the surface of the membranes of the cisternae. Unfortunately the glycogen areas were not well preserved in hydroxyadipaldehyde-fixed material, and this made it difficult to establish the relationship between glucose-6-phosphatase activity and the membranes of the smooth endoplasmic reticulum.

TABLE II

Enzymatic Activity Retained in Aldehyde-Fixed Tissue

(Estimation on the basis of light microscopy)

	SD	DP-NHD	TP-NHD	Cyto. ox. C	Cyto. ox. S	Cytopl. ester.	Cath. C	Neuro. AChE	Alk. Pase	Acid Pase	ATPase	AMP-ase	G-6-Pase
Glutaraldehyde	0	M	M	M	0	M	M	I	I	M	M	M	0
Glyoxal	M	M	M	I	M	M	—	M	I	0	I	M‡	0
Hydroxyadipaldehyde	I	I	I	I	I	T	0	—	I	S	I*	M	M
Acrolein	0	0	0	0	0	T	M	0	S	O	0	0	0
Methacrolein	0	I	I	M	0	M	0	—	I	M	I	M‡	0
Crotonaldehyde	T	I	I	0	0	M	0	—	I	M	I	T	0
Acetaldehyde	0	I	I	M	S	M	0	—	I	S	I*	M‡	—
Pyruvic aldehyde	T	0	0	M	T	0	0	—	I	S	I*	M‡	—
Formalin	0	I	I	S	T	M	M	I	I	M	I	M	0

I, intense; M, moderate; S, slight; T, trace; 0, no activity.

Enzyme activities studied are: SD, succinic dehydrogenase; DPNHD, reduced diphosphopyridine nucleotide diaphorase; TPNHD, reduced triphosphopyridine nucleotide diaphorase; cyto. ox., cytochrome oxidase both with (C) and without (S) exogenous cytochrome *c*; cytopl. ester., cytoplasmic esterase (presumably aliesterase); cath. C, esterolytic activity, resistant to E 600, occurring in cytoplasmic bodies, presumably cathepsin C, neuro. AChE, esterolytic activity confined to the myoneural junction and sensitive to eserine or DFP; alk. Pase, alkaline phosphatase; acid Pase, acid phosphatase; ATPase, adenosine triphosphatase, * cytoplasmic reaction as well as membranous; AMPase, 5-nucleotidase, ‡ cytoplasmic reaction; G-6-Pase, glucose-6-phosphatase.

DISCUSSION

Though in this discussion we should like to make several general points, it is obvious that the final decision concerning the use of these and similar aldehyde fixatives should be reserved until more

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widespread experience is gained. Concerning the mechanism of action of some of these compounds, it should be noted that the dialdehydes are excellent cross-linking agents that react rapidly, especially with active hydrogen, amino, and imino groups in protein and hydroxyl groups of polyalcohols (16–20). This cross-linking property results in the *in situ* insolubilization of many proteins and gives what can be regarded as a relatively undistorted fixation of cellular structures, which, especially in the case of glutaraldehyde, was superior to that obtained with formalin. Compounds with two aldehydes in the same molecule not only may react with the same groups in protein as compounds with one aldehyde, but, more importantly, in doing so will at the same time establish the intermolecular bridges that in the case of formalin result from the slow process of formation of methylene bridges by a two-step reaction (54). The possibility exists that dialdehydes of from 2 to 6 carbon chain lengths produce different spatial arrangements in the cross-linking between adjacent or even nearby protein chains. Although we have not yet tested them, it would be worth while to investigate the dialdehydes of succinic and fumaric acid, as well as some of the larger chain dialdehydes, as fixatives.

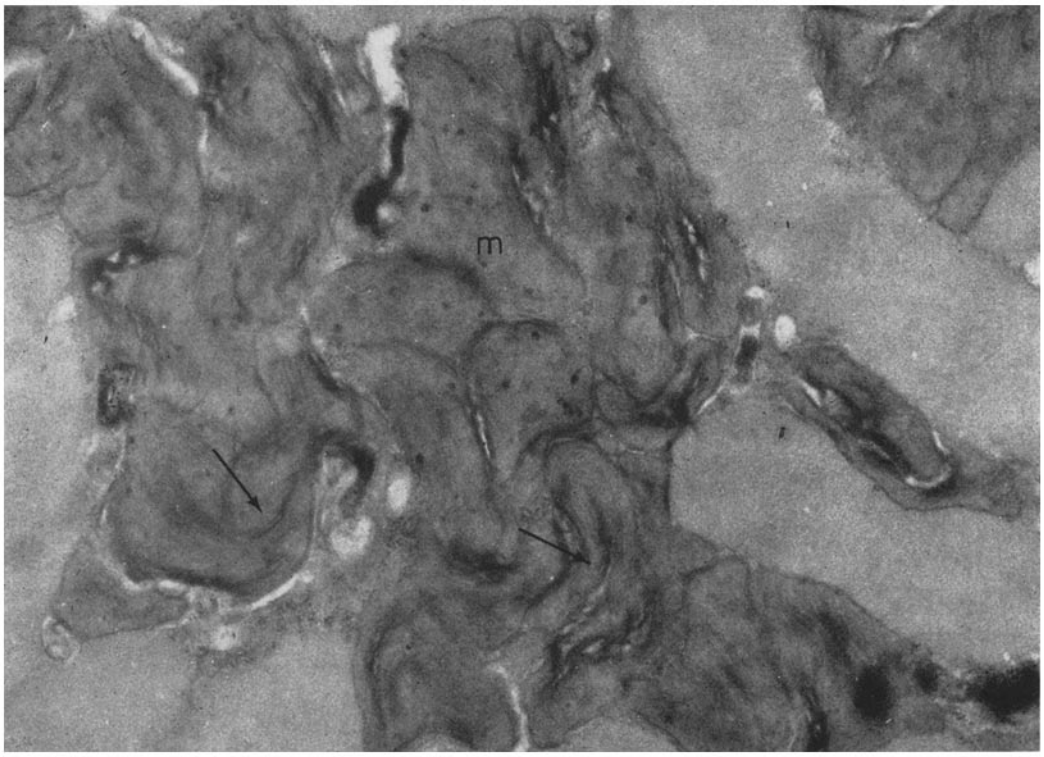

FIGURE 24

Rat myocardium fixed in hydroxyadipaldehyde, reacted for the demonstration of succinic dehydrogenase activity and briefly refixed in osmium tetroxide. Dense deposits of the final product (diformazan of nitro BT, non-metal containing organic molecule) of various sizes (arrows) overlie mitochondrial (*m*) membranes and cristae. × 30,000.

Hydroxyadipaldehyde did not have the reactivity of and did not penetrate as rapidly as glutaraldehyde or glyoxal, presumably because of the size of the dialdehyde or the molecular configuration. Though glyoxal penetrates rapidly, it is a short molecule with less possibility for cross-linking separated protein chains. Glutaraldehyde, probably assuming a shorter length and a cyclical configuration, could be accommodated between reacting protein groups separated by a distance

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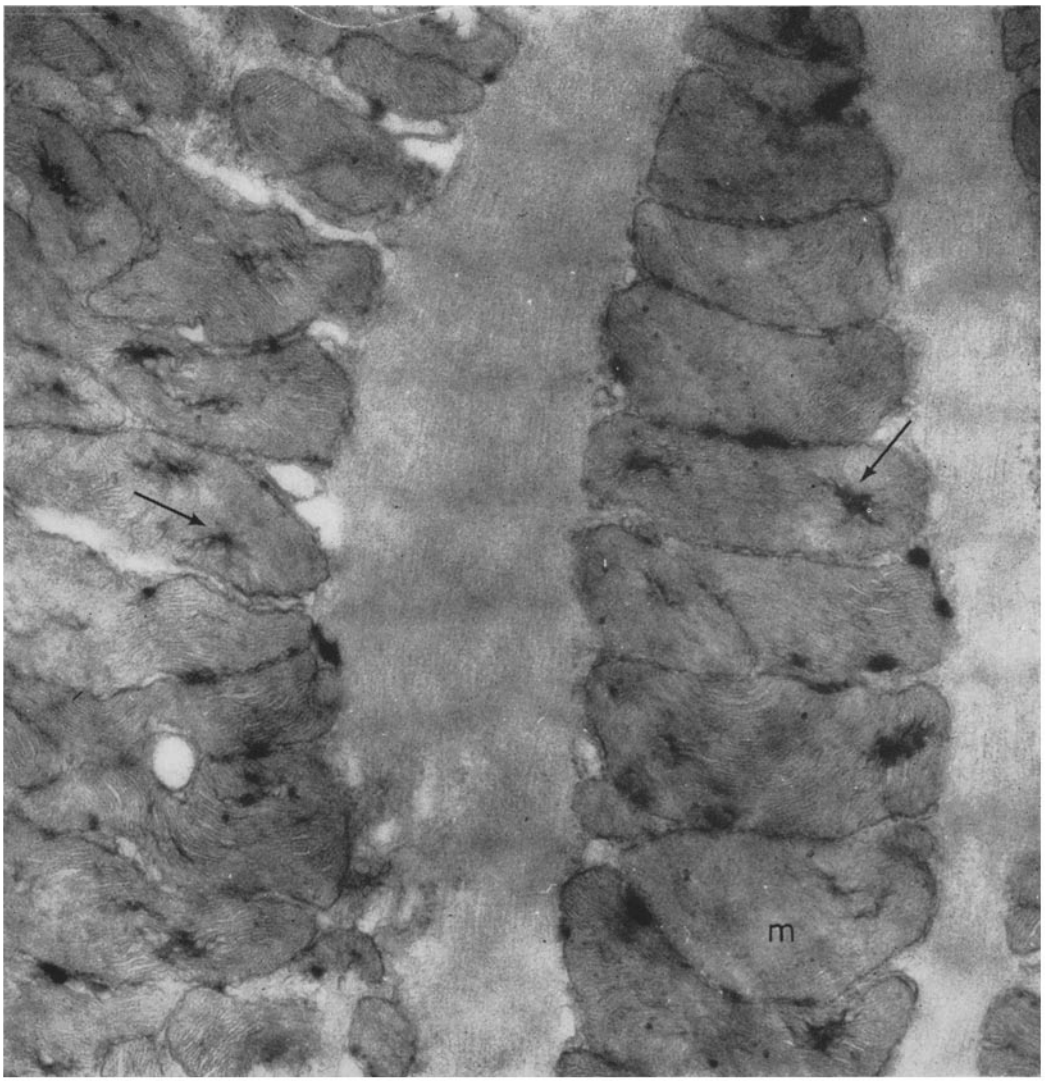

FIGURE 25

Rat myocardium fixed in hydroxyadipaldehyde and reacted for the demonstration of cytochrome oxidase activity before refixation in osmium tetroxide. Note the preservation of mitochondrial structure (*m*) and the presence of dense organic final product (arrows) irregularly distributed within or on the surface of the organelles. × 30,000.

less than the original pentamic chain. The reaction of each carbonyl group of the dialdehydes with groups in protein could be equivalent to that of formalin, and consequently methylene bridges also could occur, given sufficient time.

Concerning the monaldehydes, acrolein, as pointed out by Luft (15), is one of the most reactive organic substances. Unfortunately, this is a very noxious agent which is difficult to handle. In addition to reacting rapidly with a greater variety of chemical groups than the other aldehydes, acrolein blocks sulfhydryl groups by reaction of its double bond. While the aldehyde groups of the monoaldehydes will also react with sulfhydryl groups to form a hemithioacetal, this compound is unstable and the reaction easily

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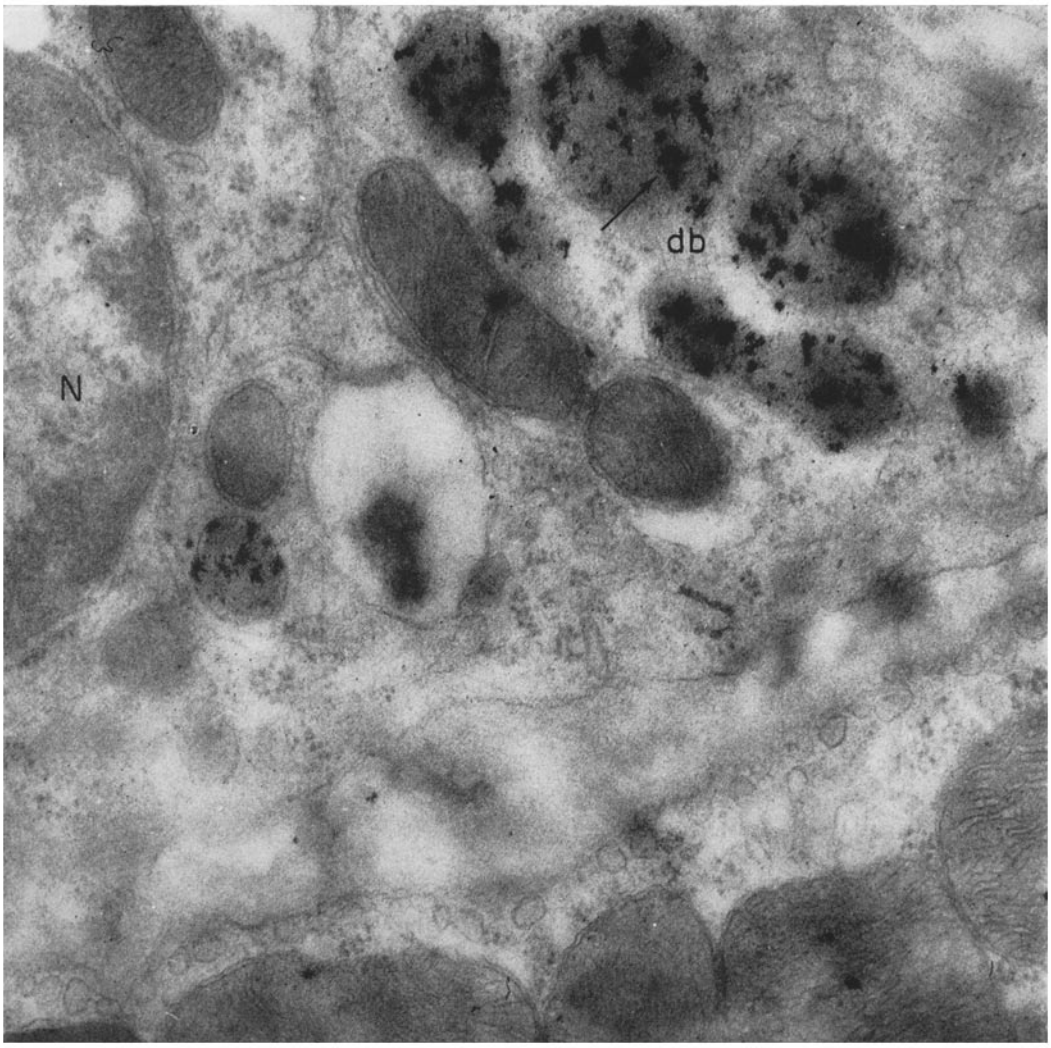

FIGURE 26

Part of a diaphragmatic muscle cell showing in the vicinity of the nucleus (*N*) several dense bodies containing the product (arrow) of a hydrolytic activity toward thiolacetic acid which is presumed to be cathepsin C. This tissue was fixed in glutaraldehyde prior to incubation and refixed in osmium tetroxide. Dense bodies, *db*. × 35,000.

reversible in aqueous solution. In assessing the other monoaldehydes, we were not looking for better fixatives than the dialdehydes or acrolein. The cross-linking ability of the former substances would not be expected to be better than that of formalin. However, a different reactivity could be expected for each particular compound, depending on the rest of the molecule, which would determine the accessibility and reactivity of the carbonyl group. As might be predicted, methacrolein and crotonaldehyde were slightly better fixatives than formalin, presumably because they contain a double bond. Pyruvic aldehyde (methyl glyoxal) and acetaldehyde, being less reactive than formalin, are poorer fixatives. Our remarks concerning formalin should not be considered as disparaging the effectiveness or use of that reagent in electron microscopy. In fact, during the course of these studies, it became quite clear that very satisfactory results could be obtained with this alde-

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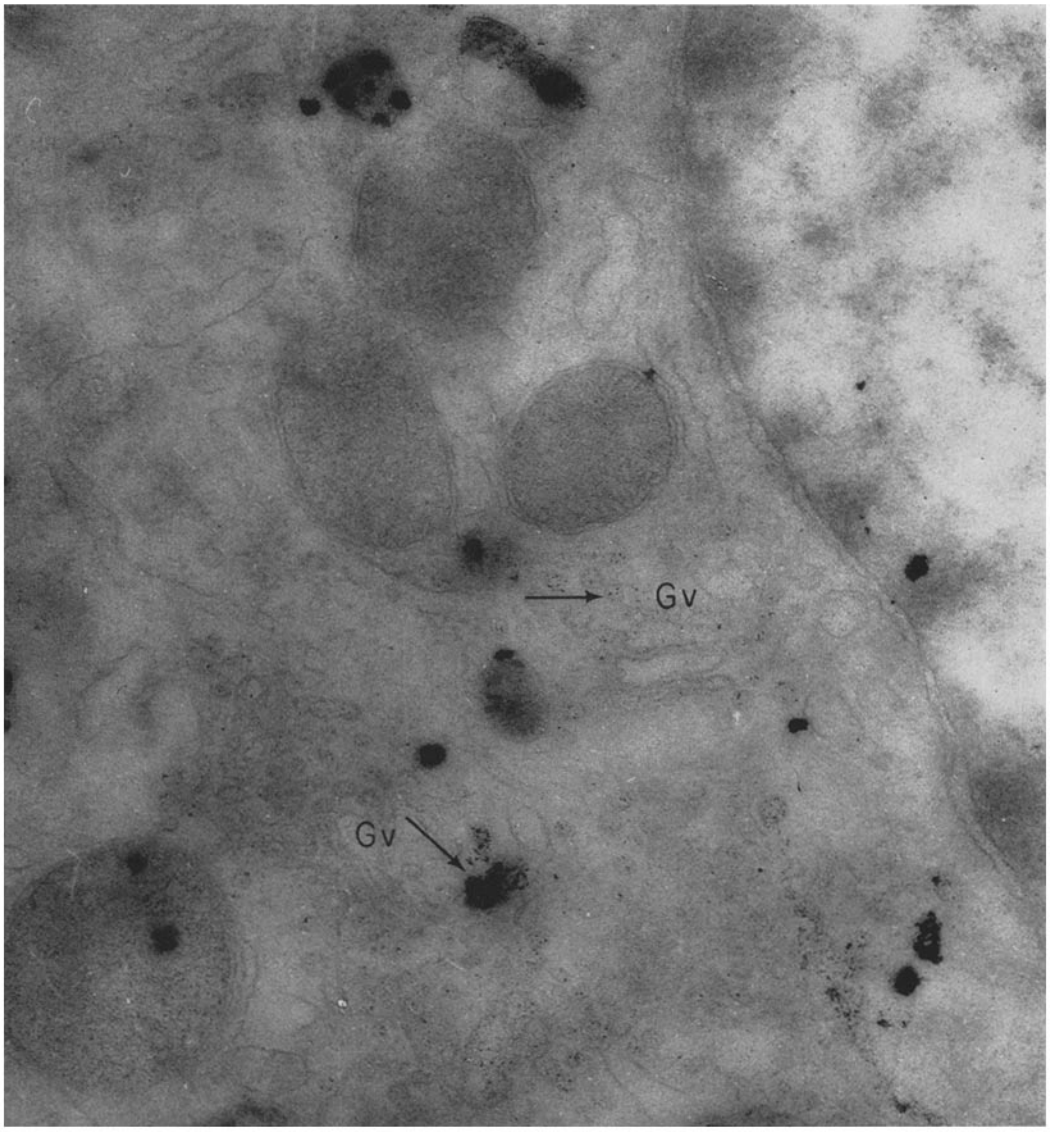

FIGURE 27

Golgi region of a distal tubular cell of rat kidney fixed in glutaraldehyde and reacted with thiolacetic acid in the presence of Pb^{++}. Golgi vesicles (*Gv*) contain a fine precipitate of final product (arrows), and in the same region the small dense bodies contain coarse granules. This reaction is not inhibited by E 600 and may be due to the lysosomal cathepsin C. × 45,000.

hyde followed by a second fixation in osmium tetroxide, provided the formalin is buffered to pH 7.4, the osmolar concentration of the fixing solution is increased with sucrose, and, most especially, the fixed tissues are embedded in Epon. Similar findings concerning the use of this fixative have been reported previously by other authors (12–14) who applied this substance to cytochemical studies with the electron microscope. According to our experience, some of the difficulties noted in the previous use of this fixative can be attributed to severe sublimation with methacrylate as embedding medium caused by the electron beam.

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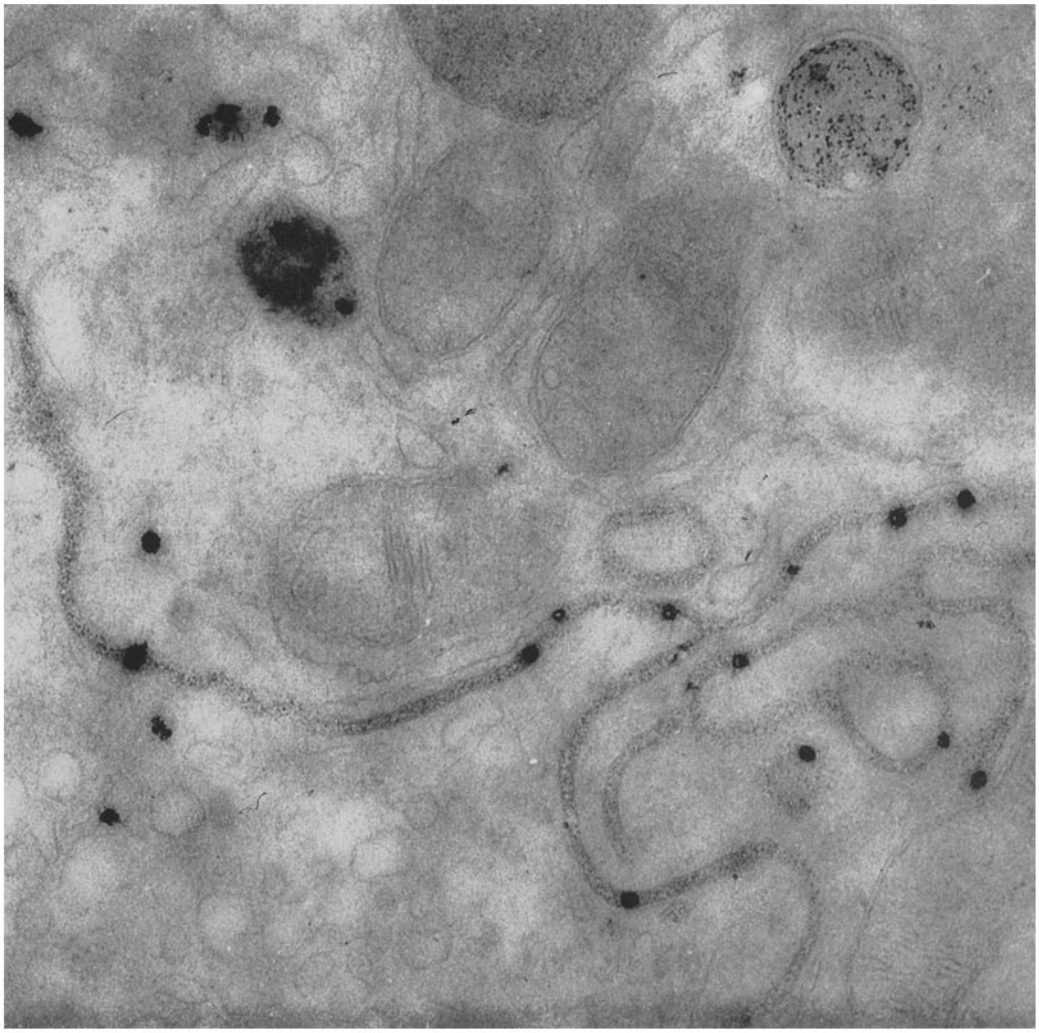

FIGURE 28

Rat kidney fixed and reacted in block as in Figs. 26 and 27. A precipitate is observed on the dense bodies and also on the cell membranes of the basal infoldings of distal convoluted tubule cells. The significance of this last localization is not clear, since, though the reaction is inhibited by E 600, it is always very faint or absent when the incubation is performed on thick frozen sections. × 45,000.

Material fixed in an aldehyde alone resembled in electron opacity that prepared by freeze-drying (44, 45), but the former appeared clearly superior in both the quality and the amount of discernible cytological detail. Both means of preparation largely fail to reveal the presence of membranous elements. In the case of the aldehyde-fixed material, this may be due to the low density of the lipid components of the membranes, which could be made apparent by staining of the section with osmium tetroxide. On the contrary, in the frozen-dried material stained with osmium tetroxide only structures reminiscent of mitochondrial cristal membranes can be distinguished, probably because most lipid components are extracted during embedding. Although frozen-dried or frozen-substituted material studied by other authors has not allowed sufficient resolution to be obtained for critical study of nuclear fine structure, the present results are in agreement with

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the gross findings, *e.g.*, peripheral and perinucleolar disposition of dense chromatin mass and of nuclear pores.

Up to the present time most of the fine structural details of cells revealed by the use of osmium tetroxide as a fixative (3) have been confirmed by the use of potassium permanganate (4) (excepting nuclear details, ribonucleoprotein particles, and and vesicles in the Golgi zone). On the other hand, most membranous structures of the cytoplasm (plasma, nuclear, Golgi, and endoplasmic reticulum membranes as well as the outer mitochondrial membranes) were lacking. However, when aldehyde-fixed material was postfixed in osmium tetroxide, a complete accordance of detail was attained for the enumerated structures. This

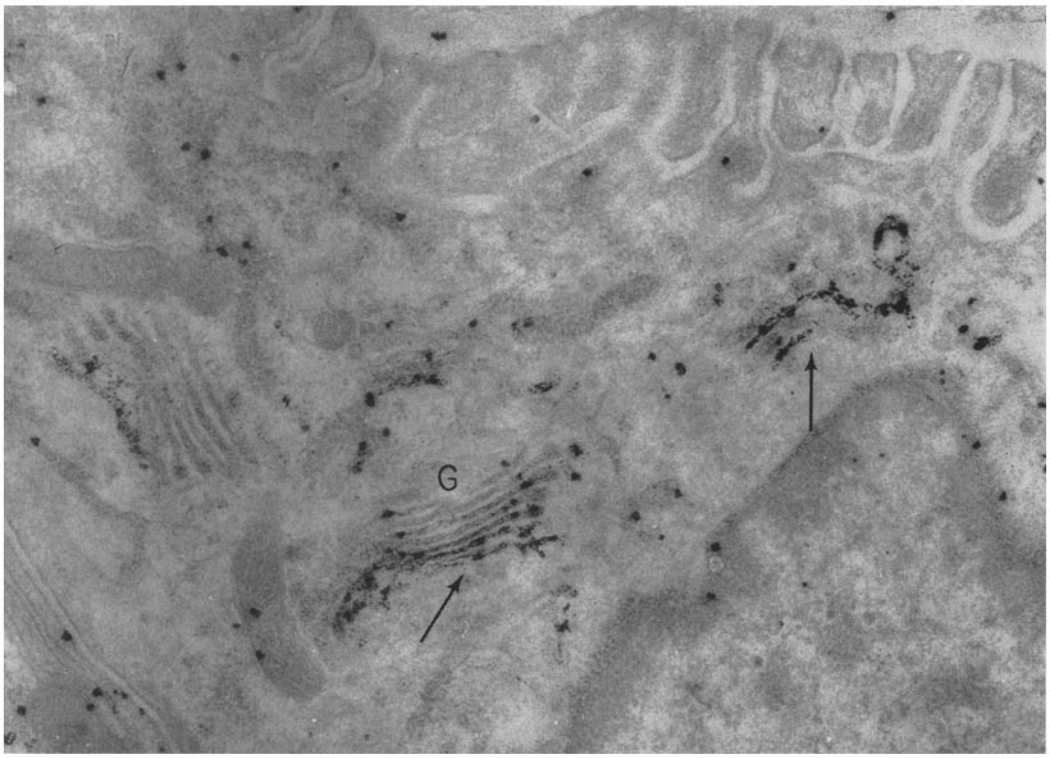

FIGURE 29

Glomerular epithelial cell subject to the same fixation and treatment as noted for the previous three figures. The final product (arrows) appears in association with several Golgi zones (*G*), outlining the parallel lamellae. A scattering of some non-specific deposits is apparent. × 20,000.

myofilaments) and to a lesser extent by freeze-drying (44, 45) or cryofixation (46, 55). Further and more direct support of the morphological information derived from osmium tetroxide–fixed cells is provided by the use of aldehydes. Though the material fixed in aldehyde shows a submicroscopic organization of the cytoplasm similar to that of material fixed in osmium tetroxide, this similarity extends only to some of the fine structural details (ribonucleoprotein particles, and presence of mitochondrial cristae and of vacuoles finding strongly supports the confidence that was put in the reality of the main features of the image that osmium tetroxide gives of cytoplasmic structure.

It is interesting that double fixation acts as a combination able to demonstrate some details of fine structure more obviously than does either fixation alone. This is the case for the particles in the glycogen areas of the rat liver. It is possible that they are not apparent after glutaraldehyde fixation because of their low density, but that this

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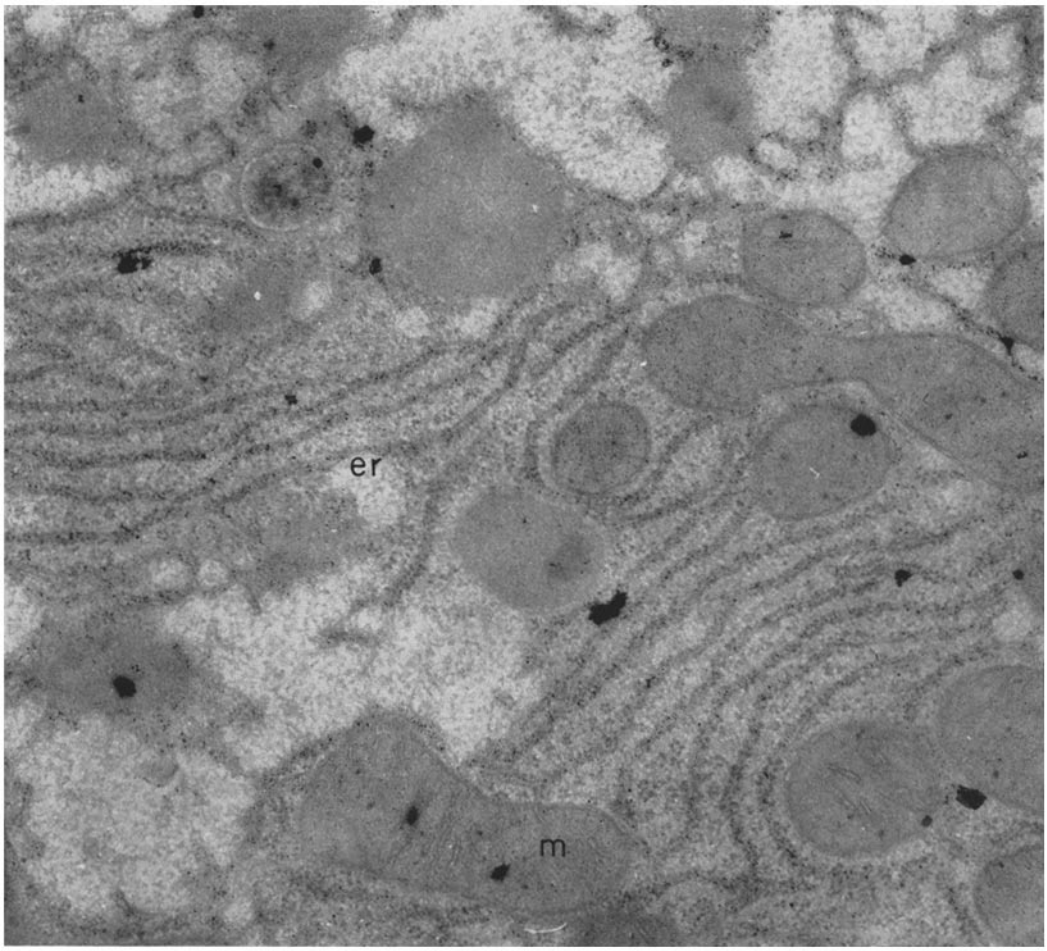

FIGURE 30

Part of a rat hepatic cell showing fine deposits in relation to the endoplasmic reticulum (*er*), indicative of a positive reaction toward the hydrolysis of thiolacetic acid. The final product is found in the rough part of the endoplasmic reticulum as well as in the agranular reticulum permeating the glycogen areas (top right). This reaction is inhibited by E 600 and is presumably due to aliesterase activity. Mitochondrion, *m*. × 30,000.

aldehyde, because of its reactivity with amino and polyhydroxy compounds, retains the material which is stained after postfixation in osmium tetroxide.

While in some cases the membranes of mitochondrial cristae were apparent after aldehyde fixation alone, outer mitochondrial membranes as well as those of the endoplasmic reticulum were not clearly discernible. Outer mitochondrial membranes could be developed by postfixation of the blocks or by staining of sections with osmium tetroxide, but this was not always the case for other membranous structures. This finding possibly reflects the specific composition of the mitochondrial membranes.

The major morphological difference between the osmium tetroxide– and aldehyde-fixed material was encountered in the nuclear structure. Postfixation in osmium tetroxide of aldehyde-fixed tissues greatly modified the nuclear structure (by dispersion or dissolution of portions of the chromatin masses) as compared with that obtained with aldehyde fixation alone. Furthermore, the chromatin masses, even though altered by double

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fixation, were more conspicuous with this procedure than with osmium tetroxide fixation alone. The gross details of the nuclear image obtained with aldehyde fixation alone are in agreement with those commonly seen in light microscopy of both fixed and frozen-dried material, and in electron microscopy of frozen-dried and variously fixed and processed tissues (48, 56–58). These facts can be taken as further evidence that osmium tetroxide is not a reliable fixative for electron microscopic studies of nuclear structure. Though we have described the fine structural details of the nucleus after glutaraldehyde fixation, this should not be taken as a "true" image of the nuclear organization, since we have seen differences in the details of the nuclear structure with different aldehydes, and insufficient work has been done in this area.

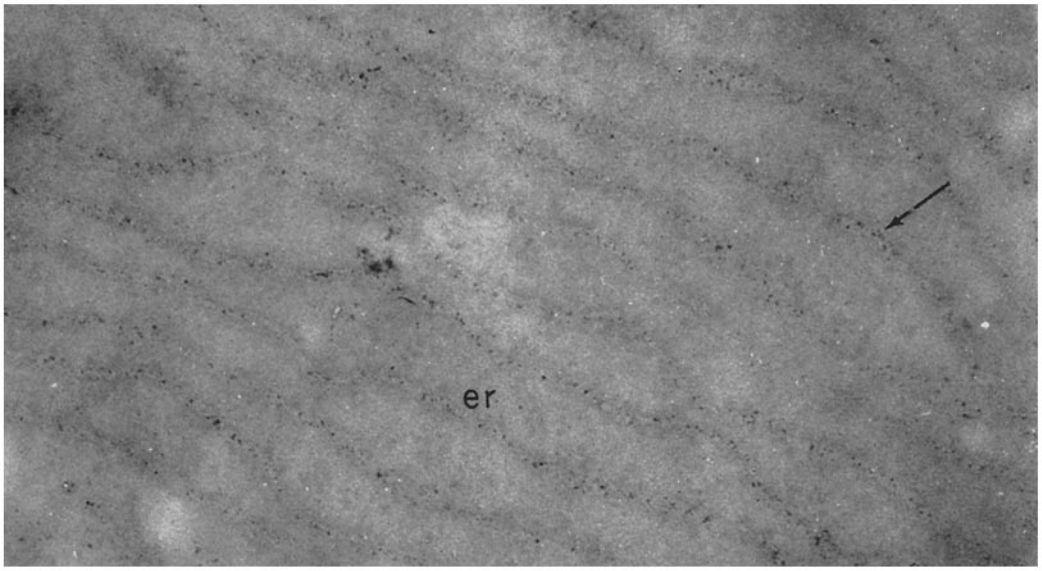

FIGURE 31

Same as Fig. 30, showing distribution of final product within the cisternae of the endoplasmic reticulum (*er*). Deposits of final product are indicated by arrow. × 55,000.

The appearance of the nucleolus in aldehyde-fixed material before and after lead staining deserves emphasis. In unstained material, the nucleolus is of homogeneous low density. However, lead solutions stain the nucleolus and bring out the presence of dense granules of smaller size (~ 100 A) but similar in appearance to the ribonucleoprotein particles found in the cytoplasm of the same cell. The cytoplasmic particles, as was pointed out before, are also intensely stained by the same procedure. The worth of investigating different electron images produced by different means of fixation is further emphasized by the fact that osmium tetroxide fixation completely masks a conspicuous finding of aldehyde fixation alone, namely, the clear channels that pass from the karyoplasm through the nuclear pores to extend well beyond the nuclear contour to the hyaloplasm.

Although some enzyme activities have been demonstrated to survive brief fixation in either osmium tetroxide or potassium permanganate (5–9), only after aldehyde fixation was a large variety of enzyme methods applicable to fixed tissue that could be processed after days or weeks of buffer storage.

The achievement of discrete localization of products of enzymatic activity at the submicroscopic level relates primarily to two factors in the present work: the preservation of fine structural details obtained through fixation, and the maintenance of sufficient enzyme activity by a proper choice of fixative. However, there are additional factors to be considered. While the fixation is done immediately, the operational procedure for histochemistry and electron microscopy can be

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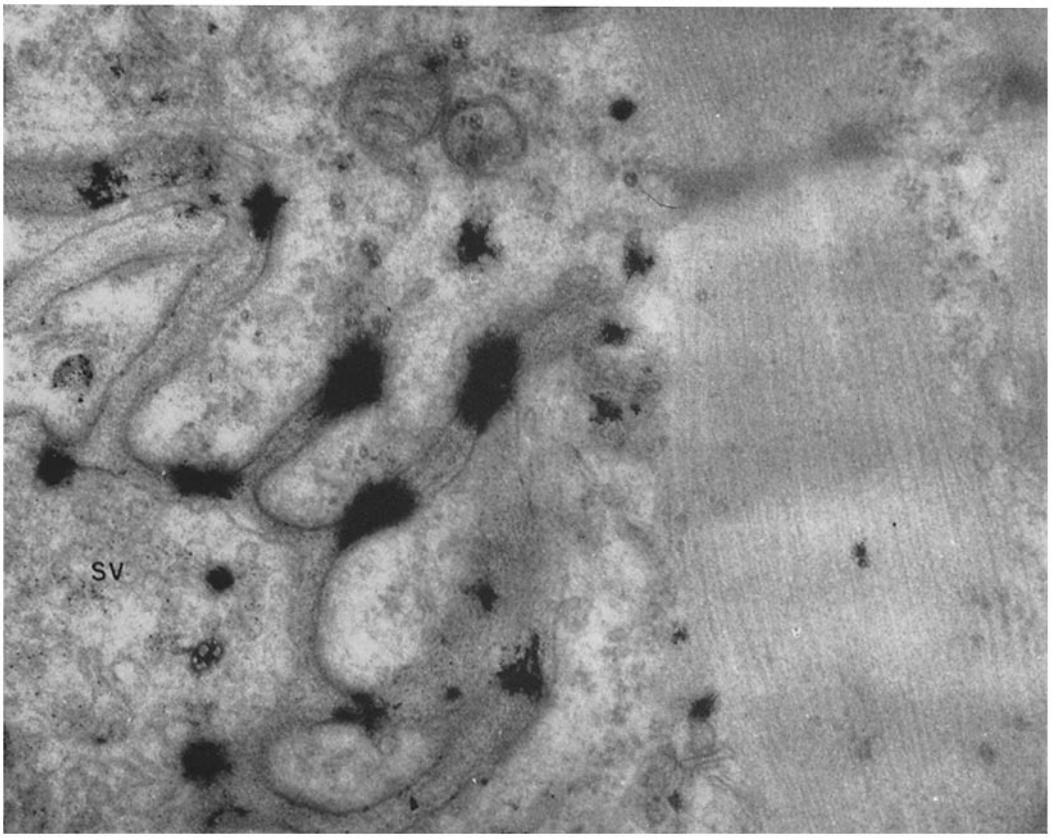

FIGURE 32

Acetylcholinesterase activity of myoneural junction demonstrated by the thiolacetic acid method. Spotty localization occurs in the primary and secondary synaptic clefts of this glutaraldehyde-prefixed material, and fine deposits can be observed in the synaptic vesicles (*sv*). The activity is completely inhibited by eserine and DFP (10^{-4} M). × 36,000.

rather leisurely delayed, by storing fixed tissues in cold buffer. In addition, fixed tissues suffer less than fresh tissue from the possibility of the migration of soluble and diffusible enzymes. The resulting decrease in the activity of the enzymes produced by fixation allows a more immediate capture of the product by the reagent and a discrete localization of final precipitate.

Since the aim of the present work was to introduce the use of aldehydes for histochemistry and electron microscopy, the study of the distribution of the various enzymatic activities will be found incomplete. Some of the localizations, however, are in accordance with previous work and with what is now known concerning the presence of enzyme activities in the different cell compartments. For example, the products of several oxidative enzyme activities were primarily related to mitochondrial membranes (59), while acid phosphatase (14) and presumably cathepsin C activities were demonstrable in dense bodies (lysosomes). Microsomal enzymes such as glucose-6-phosphatase (41) and aliesterase (60) assayed histochemically gave a product clearly confined in the liver to the membranes of the endoplasmic reticulum. More difficult to interpret is the localization of the product of hydrolysis of thiolacetic acid in relation to the cytoplasmic membranes of the kidney cells. Since the incubation of thick frozen sections resulted in little or no activity in relation to the membranes of the kidney, the possibility exists of a complicated situation of diffusion gradients inside the small blocks, resulting in a fallacious localization of the precipitate.

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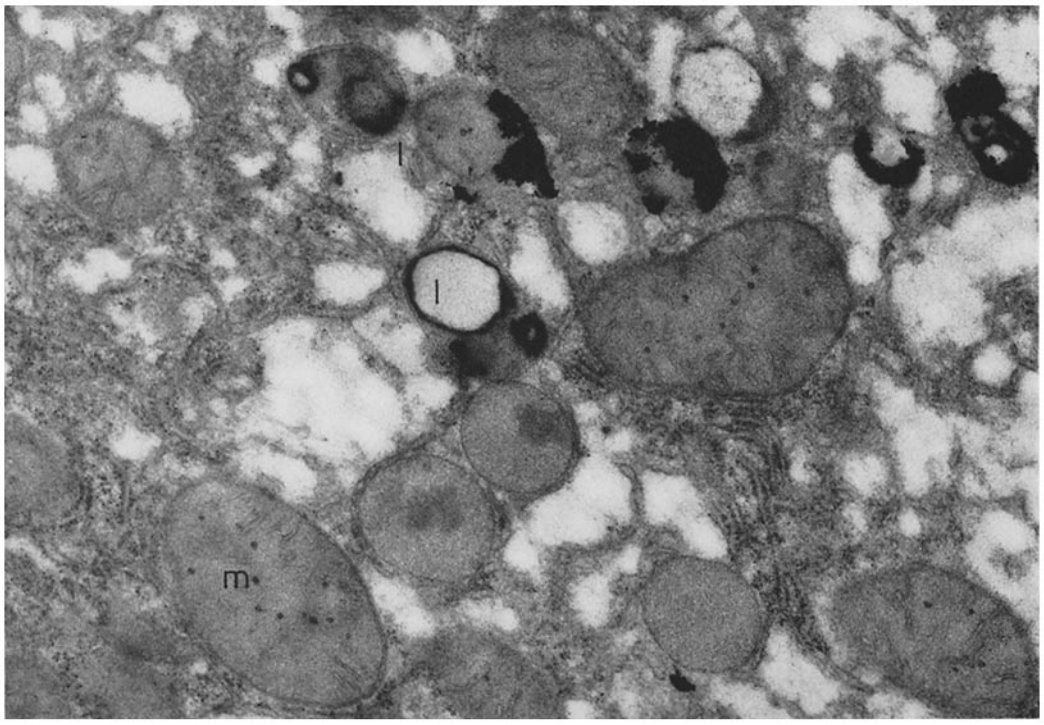

FIGURE 33

Several peribiliary bodies (lysosomes, *l*) show lead phosphate deposits indicative of acid phosphatase activity. Rat liver fixed in glutaraldehyde. Mitochondrion, *m*. × 25,000.

These points raise the question of the use of thick frozen sections or of small blocks for electron cytochemistry. The tests reported in this paper were duplicated with both techniques, and congruent results were obtained in all cases with the exception noted previously. However, with the techniques for the phosphatases, the non-specific deposits are fewer in sections than in blocks, and the former should be preferred in spite of some damage to the structure.

The fact that some fixatives preserved the activity of succinic dehydrogenase, cytochrome oxidase, and glucose-6-phosphatase should be

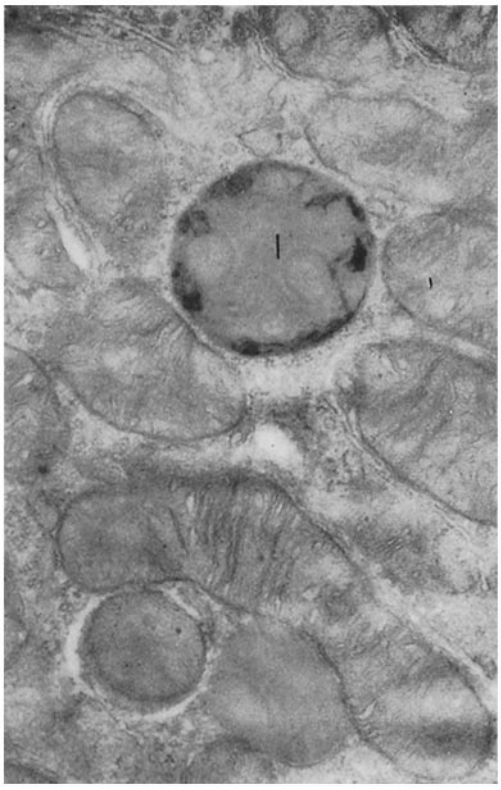

FIGURE 34

Part of tubular cell of the rat kidney fixed in glutaraldehyde, showing acid phosphatase activity restricted to a lysosome (*l*). This activity was demonstrated by a 15-minute incubation period. Longer incubation times produce still heavier deposits confined to these structures. × 28,000.

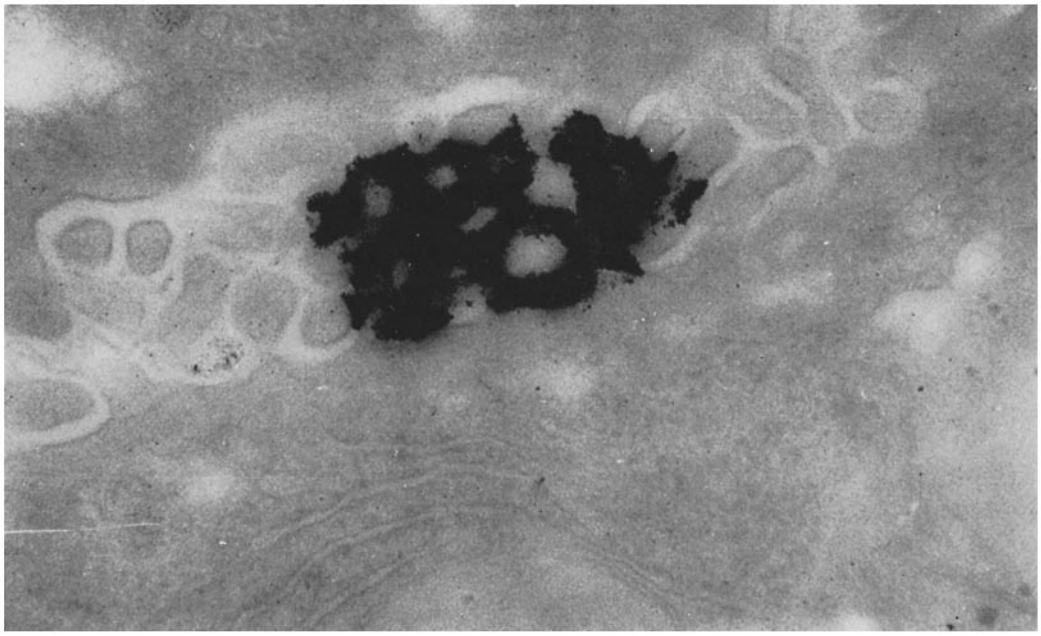

FIGURE 35

Rat liver fixed in crotonaldehyde and incubated for the demonstration of ATPase activity. The biliary canaliculus is filled with the dense final product of the reaction. Other parts of the periphery of the liver cell, *e.g.* that facing the sinusoid, also showed some activity. × 35,000.

stressed since these enzymes heretofore have not survived routine fixation. However, it should be noted that hydroxyadipaldehyde, which best preserved the activity of these most sensitive enzymes, was not the fixative of choice for the retention of fine structural detail. This reinforces the general notion that excellent morphological preservation by fixation implies the suppression of enzyme activities. However, there is still room for choice of fixative, and a compromise can be attained between a perfect general fixation with no enzymatic activity and the retention of some activity in relation to a recognizable fine structure. It is also possible that aldehyde fixation may be applicable to studies of immunohistochemistry in which macromolecules must retain their antigenic properties.

A singular advantage of using appropriately fixed material in the combination of histochemistry with electron microscopy comes from the fact that a similar quality of morphological preservation found with aldehyde fixation alone was maintained through the incubation procedures and was present in the final analysis with the electron microscope. In addition, the preservation of morphology was sufficiently good with some of the aldehydes alone (*e.g.* glutaraldehyde) so that the final dense product could be easily referable to fine structure in sections of material embedded without postosmication. This fact suggests the possibility that low density final products of histochemical reactions might also be recognized in material fixed in aldehyde alone.

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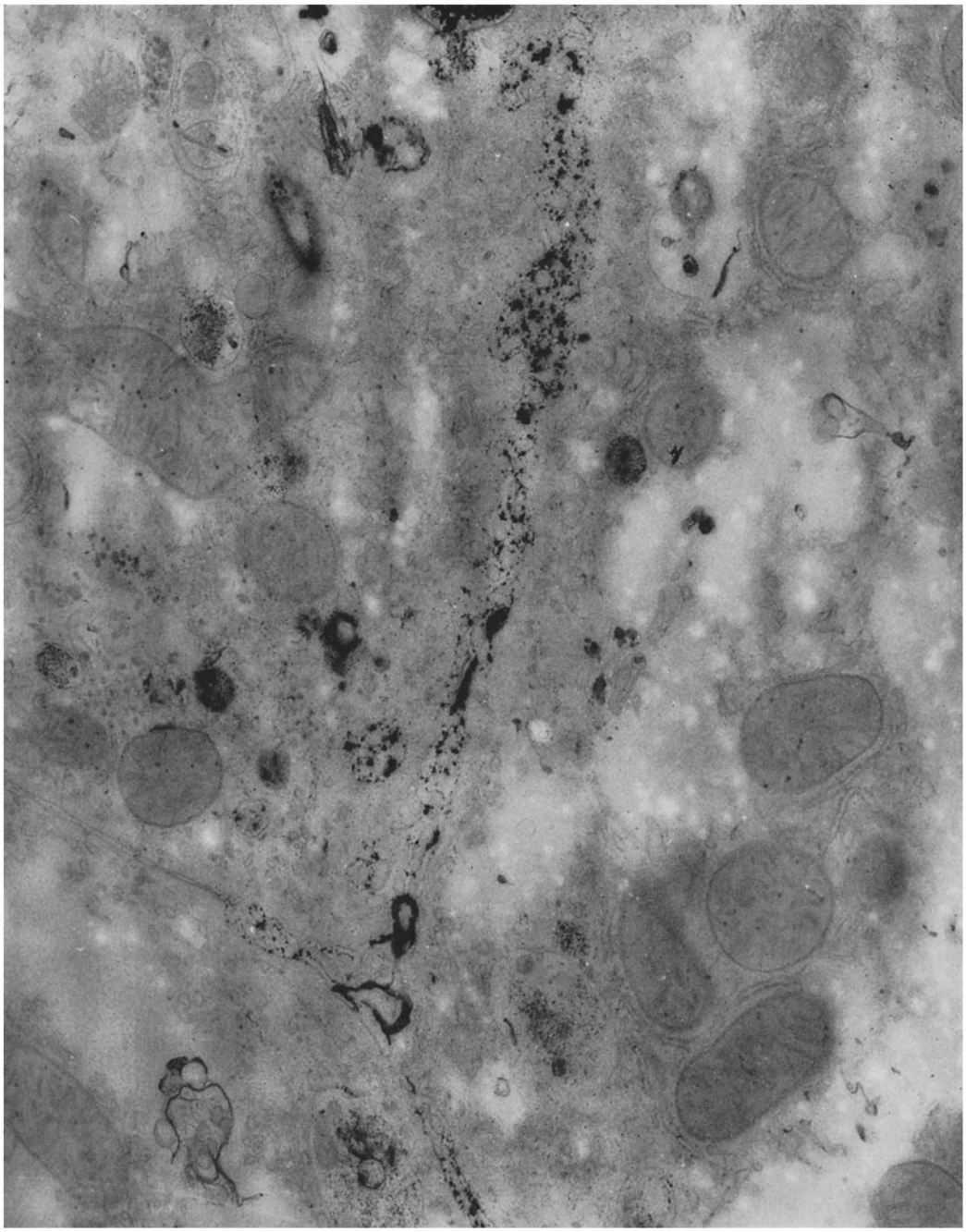

FIGURE 36

Liver tissue fixed in glutaraldehyde and incubated to demonstrate 5-nucleotidase activity. Parts of three hepatic cells are shown with the dense product localized in the cell borders and intercellular space. The activity which in the peripheral dense bodies also hydrolyzed the substrate, AMP, presumably is due to acid phosphatase. A myelin figure at the bottom left is stained by the lead in the incubating medium. × 31,000.

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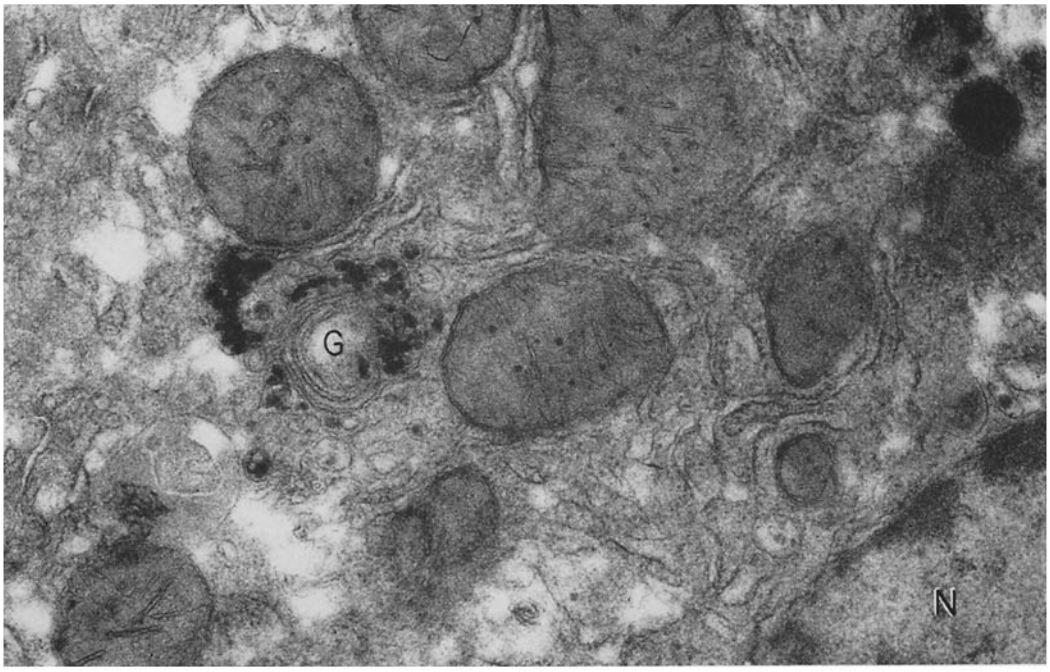

FIGURE 37

Glutaraldehyde-fixed hepatic cell incubated for the demonstration of a nucleoside-diphosphatase activity with IDP as substrate. Sacs in the Golgi region (*G*) contain the final dense product (lead phosphate). Nucleus, *N*. × 35,000.

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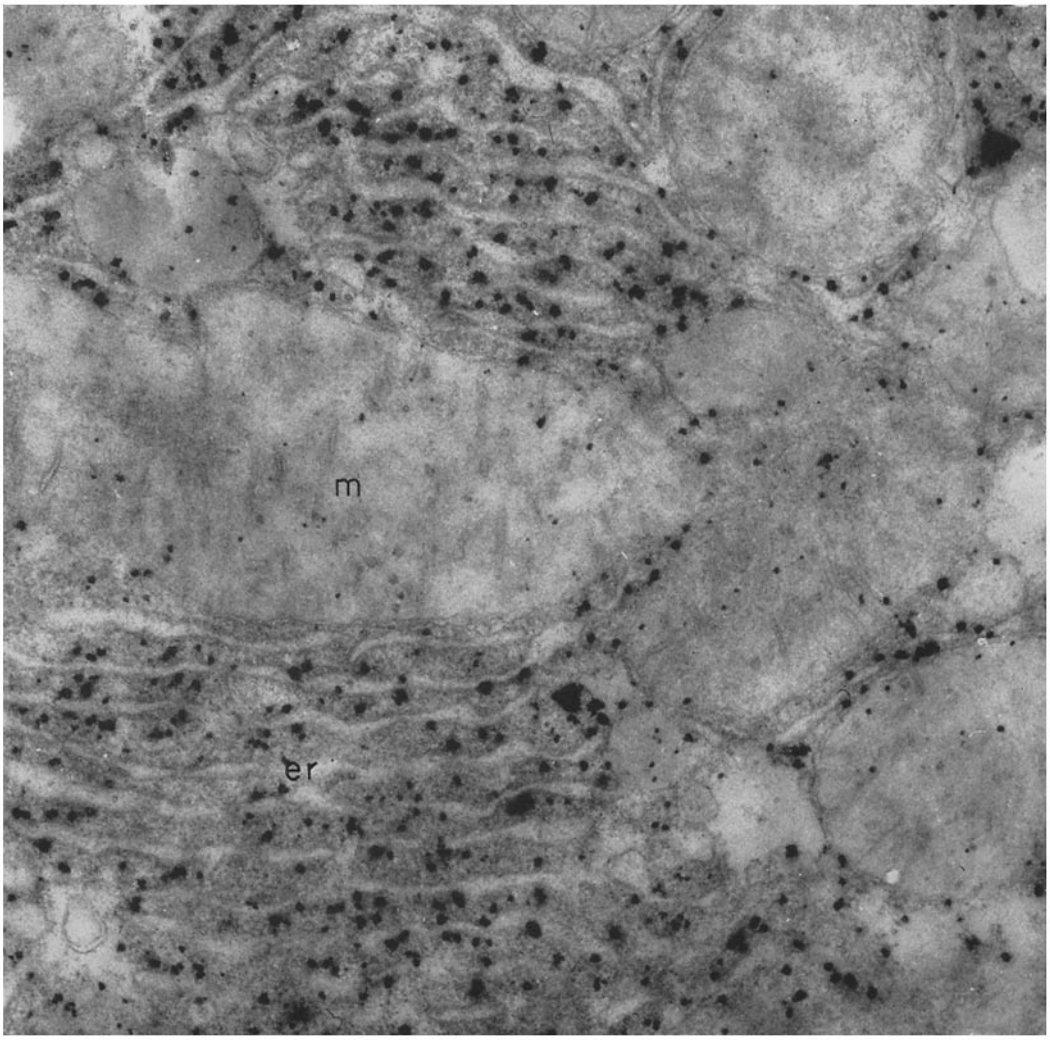

FIGURE 38

Rat liver fixed in hydroxyadipaldehyde and incubated for the presence of glucose-6-phosphatase activity. The final product is located in relation to the cisternae of the endoplasmic reticulum (*er*). Mitochondria (*m*) show no product. × 40,000.

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