

THE ULTRASTRUCTURAL LOCALIZATION OF THE ISOZYMES OF ASPARTATE AMINOTRANSFERASE IN MURINE TISSUES

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ABSTRACT

Two isozymes of aspartate aminotransferase have been demonstrated biochemically. One isozyme is found in the mitochondrial fraction of the cytoplasm, the other ("soluble") in the supernatant. Both isozymes can be demonstrated by the cytochemical technique of Lee and Torack, as reported in the preceding report. Aldehyde fixation rapidly inactivates both isozymes, especially the soluble one. Inactivation can be delayed by addition of ketoglutarate to the fixative. The ketoglutarate probably competes with the fixative for the active site of the enzyme, thus protecting that region of the molecule. This enables adequate tissue preservation with enough remaining enzymatic activity to be demonstrated by the precipitation of oxaloacetate as the lead salt from a medium containing α -ketoglutaric acid aspartic acid, and lead nitrate. Electron-opaque material was found not only in mitochondria but, as the result of substrate protection, on the plasma membranes of many cells including erythrocytes and bacteria, the limiting membrane of peroxisomes, and the transverse tubular system of striated muscle. Occasional centrioles, neurotubules, tubules in the tails of spermatozoa, the A-I band junction in myofibrils of striated muscle, and the ground substance between cisternae of endoplasmic reticulum in intestinal goblet cells also showed precipitate. In all cases, replacement of L-aspartic acid by D-aspartic acid in the medium resulted in unstained sections. The sensitivity of extramitochondrial sites to fixation, the need of ketoglutarate as an agent for protecting the enzymatic activity during the fixation process, and the known presence of only soluble isozyme in erythrocytes indicate that enzymatic activity at these sites can be attributed to the soluble isozyme. Localization of the soluble isozyme on the plasma membrane may be related to possible involvement in depolarization phenomena, amino acid transport, or synthesis of plasma membrane-bound mucopolysaccharides.

INTRODUCTION

In the preceding paper (29), it was established that, in a model system containing the cytoplasmic fractions of rat liver, both the mitochondrial and the "soluble" isozymes of aspartate aminotransferase (E.C. 2.6.1.1., glutamate-oxalacetate transaminase) (2, 3, 5, 6, 7, 9, 22, 24, 25, 31) can be demonstrated by the technique recommended by Lee and

Torack (17, 19, 20). The rapid inactivation of the soluble enzyme by aldehyde fixation was diminished by the addition of ketoglutarate to the fixative (29). The ketoglutarate is thought to compete successfully with the fixative for the active site of the enzyme and thus delay inactivation. In addition, the optimum substrate concentration for de-

recting soluble enzyme activity was determined. The information obtained from the model system studies was applied to an ultrastructural investigation of the sites of activity of the aspartate aminotransferase isoenzyme in murine tissues. These findings are reported in the present report.

Earlier ultrastructural studies by Lee and Torack on the sites of aspartate aminotransferase activity had shown what was primarily the localization of the mitochondrial isozyme (18, 19, 20) in both rat heart and rat liver. Extramitochondrial activity was noted in hepatocytic peroxisomes (19), nuclear membranes (18, 19), and vesicular components of cardiac muscle (18). As regards the reaction product surrounding peroxisomes, Lee and Torack (19) suggested that it might be due to the activity of the soluble isozyme. However, these extramitochondrial sites were not always consistently demonstrated. With the techniques outlined above, electron-opaque material, attributed to the activity of the soluble isozyme of aspartate aminotransferase, was unexpectedly found on the plasma membrane of many cell types, as well as on other cytoplasmic structures. It appears that this isozyme, which biochemically is found in the supernatant fraction of the cytoplasm, is in close association with structural cytoplasmic components.

MATERIALS AND METHODS

Murine liver, heart, diaphragm, cerebral cortex, intestine, and testis were selected for study. Adult albino mice were anesthetized, the thorax was opened, and the animal was perfused through the left ventricle for 2 min with either cold 1% glutaraldehyde (Fluka AG, Buchs, Switzerland) or 3.7% formaldehyde (E. Merck AG, Darmstadt, Germany) in 0.05 M imidazole (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) buffer (pH 7.2) containing 1 g of sucrose/100 ml. Small pieces of the selected tissues (0.5 mm³) were then placed in fresh fixative for a total of 7 or 12 min of fixation. In a second group of animals, the same procedure was followed but in this instance α -ketoglutarate was introduced in the fixative at a concentration of 117 mg/100 ml (8 mM).

After fixation, tissues were washed for 1 hr in cold 0.25 M sucrose solutions buffered with 0.05 M imidazole (pH 7.2) and then placed for 20 min in an incubation medium containing 4 mM of α -ketoglutarate (Koch-Light Lab., England), 20 mM of L-aspartate (Koch-Light Lab., England) and 6 mM of lead nitrate (pH 7.5). Incubation was performed at room temperature (20°-22°C). After incubation, specimens

were washed for 5 min in 20 mM of L-aspartate in 0.05 M imidazole (pH 7.3) as recommended by Lee and Torack (19). They were then postfixed in 1% osmium tetroxide dissolved in *s*-collidine buffer (pH 7.3) for 1 hr, dehydrated in graded solutions of ethanol containing imidazole buffer (pH 7.3), and embedded in Epon. Sections were cut on a Reichert ultramicrotome and examined in a Siemens Elmiskop at an accelerating voltage of 80 kv. Some sections were stained with lead hydroxide; others were examined unstained.

An incubation medium prepared as outlined above but containing D-aspartic acid instead of L-aspartic acid provided for a series of controls.

For light microscopy, frozen sections of liver and muscle were cut at a thickness of 10-15 μ , fixed for 2 min in cold 3.7% formaldehyde, and incubated in the medium described above. After incubation, they were treated for 5 min with a 2% sodium sulfide solution, washed, and mounted in "Aquamount" (Edward Gurr Ltd., London S.W. 14, England).

RESULTS

Generally, tissues fixed in 1% glutaraldehyde containing ketoglutarate regularly exhibited evidence of enzymatic activity, whereas the tissues fixed in glutaraldehyde from which ketoglutarate was omitted gave inconsistent demonstration of the reaction product, especially in extramitochondrial sites of enzymatic activity. An acceptable degree of ultrastructural preservation was attained in most cases, despite the rather short fixation periods employed.

Fixation with formaldehyde gave similar results. Again, preservation of enzymatic activity was greater when ketoglutarate was included in the fixative. Moreover, the combination of formaldehyde and ketoglutarate preserved more sites of enzymatic activity than the combination of 1% glutaraldehyde and ketoglutarate. Unfortunately, the ultrastructure was generally poorly preserved when formaldehyde was used, especially when the fixation times were short.

Liver

LIGHT MICROSCOPY: Brown reaction product (lead sulfide) was visible in all hepatocytes. Deposition of the reaction product was maximal in the periportal areas of the liver lobule (Fig. 1) and least in hepatocytes surrounding the central vein.

ELECTRON MICROSCOPY: Reaction product was easily recognized by its electron opacity and was seen to localize in the majority of mitochondria

within hepatocytes and sinusoidal cells (Fig. 2). Some mitochondria, especially in tissues fixed without substrate protection, failed to show the presence of enzymatic activity although adjoining mitochondria within the same cell possessed distinct deposits of reaction product (Fig. 2). These deposits were confined to the region between the inner and outer mitochondrial membranes and extended into the cristae mitochondriales (Figs. 2, 3) Reaction product was not found within the inner mitochondrial chamber.

The limiting membrane of hepatocytic peroxisomes also possessed electron-opaque deposits of reaction product (Fig. 3). These deposits were most conspicuous in specimens fixed in the presence of α -ketoglutarate, although some were found in specimens fixed in its absence. Neither the matrix nor the nucleoid of peroxisomes showed evidence of enzymatic activity.

When ketoglutarate was included in the fixative, deposits of reaction product became obvious over the membrane lining the hepatocytic microvilli in the spaces of Disse, in micropinocytic vesicles of sinusoidal cells and occasionally parenchymal cells, and the plasma membrane of erythrocytes (Fig. 3). These deposits were somewhat more conspicuous when formaldehyde was substituted for glutaraldehyde in the fixation process. Occasionally deposits were found on the membrane lining the bile canaliculi but not in any other regions of the plasma membranes lining the interhepatocytic space.

Neither the nucleus nor the nuclear membrane of hepatocytes or sinusoidal cells possessed any trace of reaction product. The endoplasmic reticulum and the Golgi complex also failed to show evidence of enzymatic activity.

Blood Vessels

The endothelial cells lining blood vessels in the heart, diaphragm, and brain all exhibited evidence

of enzymatic activity. Apart from its appearance in the scant mitochondria of endothelial cells, the reaction product was readily found within the contents of most micropinocytic vesicles (Fig. 4). Again, this was very evident in tissues fixed in the presence of ketoglutarate. Endothelia of arterioles, capillaries, and venules all showed this type of localization. In addition, deposits were also identified in some junction regions between adjoining endothelial cells.

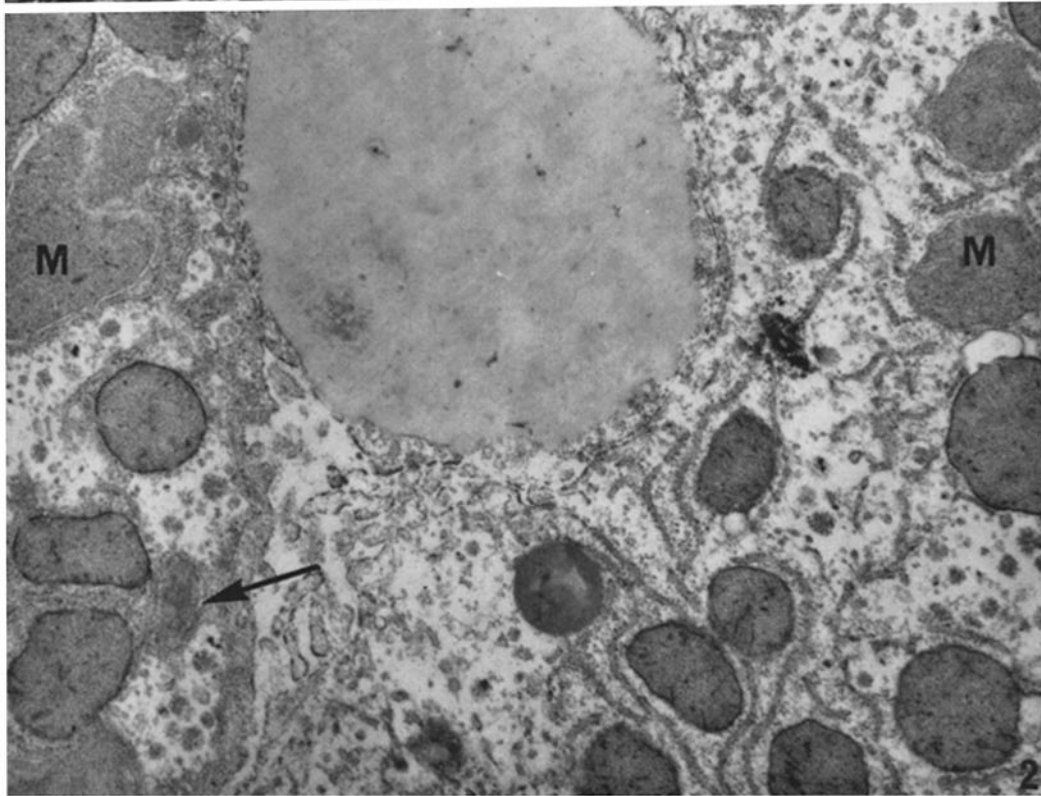
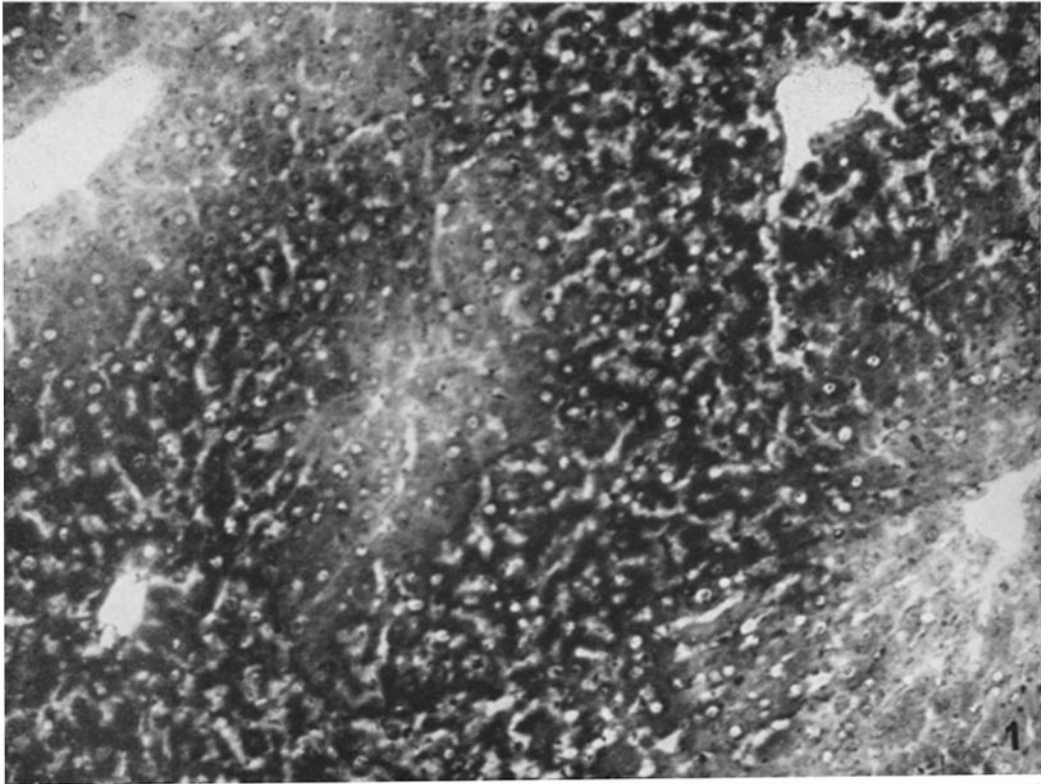
In tissues fixed for 12 min in 3.7% formaldehyde or for 7 min in 1% glutaraldehyde, but always with added ketoglutarate, deposits became obvious over the plasma membrane of endothelial cells (Fig. 5). Erythrocytes in the lumen of these vessels also showed deposits on the cell membrane (Fig. 5). Pericytes and smooth muscle cells also exhibited reaction product within micropinocytic vesicles and over cell membranes.

Cardiac and Skeletal Muscle

Again, mitochondria between myofibrils or grouped in the subsarcolemmal region showed the presence of reaction product (Fig. 6). The deposits were localized over the outer mitochondrial chamber and extended into the cristae mitochondriales. In addition, enzymatic activity was manifest around transverse tubules (Fig. 6) but not in the lumen of the tubule. These deposits were conspicuous only when ketoglutarate was incorporated during fixation. In all formaldehyde- and a few glutaraldehyde-fixed tissues, reaction product was obvious over the sarcolemmal membranes (Fig. 7) and the plasma membranes of adjoining fibroblasts (Fig. 8), provided ketoglutarate was present in the fixative. Direct connections between sarcolemma and transverse tubules were not preserved in the preparations. It was thus not possible to examine the enzymatic localizations at these sites. The region of the A-I band junction of myofibrils also exhibited dense lead deposits. Occasional

FIGURE 1 Section of mouse liver in which the dense deposits of lead sulfide indicate sites of activity of aspartate aminotransferase. Hepatocytes in the periportal area show more activity than those in the central region of the lobule. Formaldehyde fixation. Magnification, 700 \times .

FIGURE 2 Liver fixed for 12 min in 1% glutaraldehyde. Electron-opaque deposits are present in the outer mitochondrial chamber and extend into the cristae mitochondriales. Some mitochondria (*M*) do not show evidence of activity. Peroxisomes (arrow) also fail to demonstrate enzymatic activity. Lead hydroxide. Magnification, 24,000 \times .



centrioles in fibroblastic cells showed well defined deposits within the lumina of their constituent tubular structures (Fig. 8).

Nuclei and nuclear membranes remained unstained. Neither the Golgi apparatus nor the components of the sarcoplasmic reticulum showed enzymatic activity.

Brain

Cerebral tissues were difficult to preserve well because the short fixation times used in order to avoid enzymatic inactivation failed to adequately preserve all morphological details. Nevertheless, blood vessels and neurones were moderately well preserved. As expected, mitochondria within neurones, axons, and dendrites exhibited electron-opaque deposits in a distribution similar to that for other tissues. With short fixation times (7 min) with 1% glutaraldehyde and ketoglutarate protection, enzymatic activity was detected on the surface of neuronal perikarya dendrites, the periphery of occasional myelin sheaths, but not on the limiting membrane of axons (Fig. 9). Where synapses were preserved, the reaction product was confined to the gap region of the synaptic space (Fig. 10). Occasional neurotubules showed lead deposits (Fig. 11). No other cellular component showed enzymatic activity.

Intestinal Columnar Epithelium

Mitochondrial enzymatic activity was obvious in the outer mitochondrial chamber. In tissues protected with ketoglutarate during fixation, reaction product was found along the periphery of the lining columnar cells and outlining the surface of the microvilli (Fig. 12). In goblet cells fixed in formaldehyde in the presence of ketoglutarate, enzymatic activity was detected in the small bands of ground substance between cisternae of endoplasmic reticulum in the paranuclear region, and sparsely over the mucoid secretions of the cell (Fig. 16).

Bacteria present in the gut lumen also showed the presence of enzymatic activity on their surfaces (Fig. 12). The dense lead deposits were localized not on the capsule of the bacterial cell but mainly on the cell membrane of the bacterium (Fig. 13).

Testis

Apart from the mitochondria of a variety of cell types, reaction product was present in the plasma membrane of Sertoli cells and spermatozoa (Fig. 17) but less frequently in the cell membranes of spermatids and their precursors. In addition, electron-opaque deposits were seen in some centrioles and axial tubules in the tail of spermatozoa (Figs. 14, 15). Substrate protection was necessary for the demonstration of all extramitochondrial sites of activity.

Controls

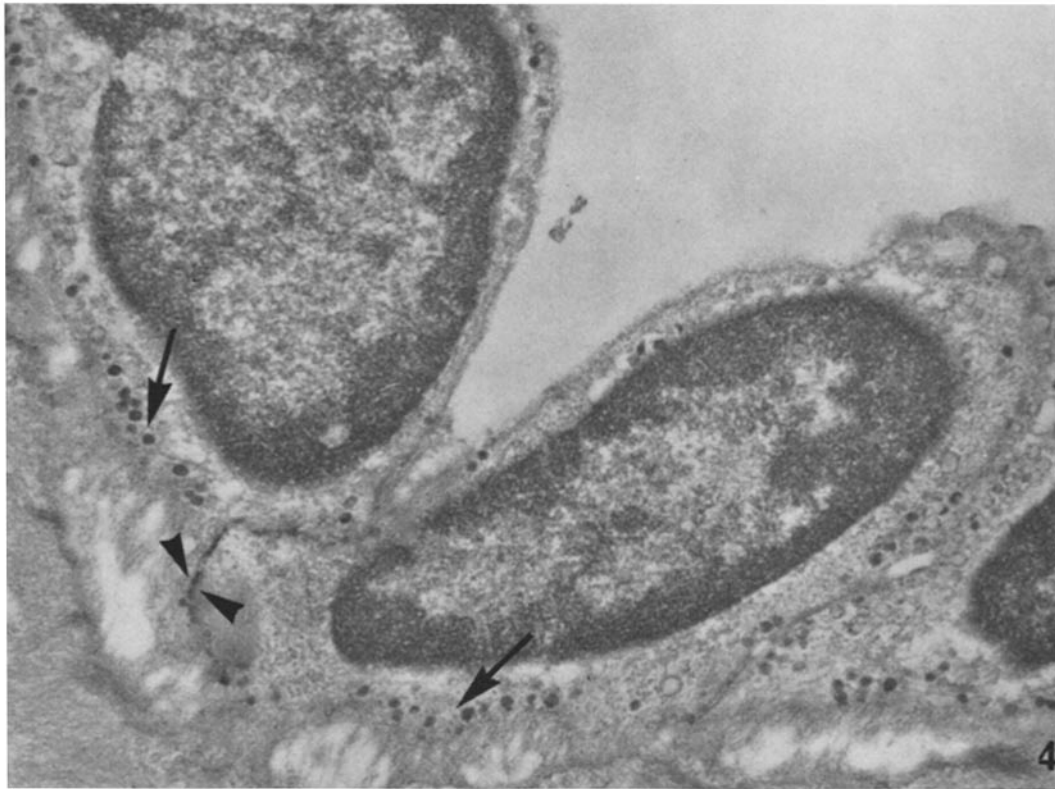
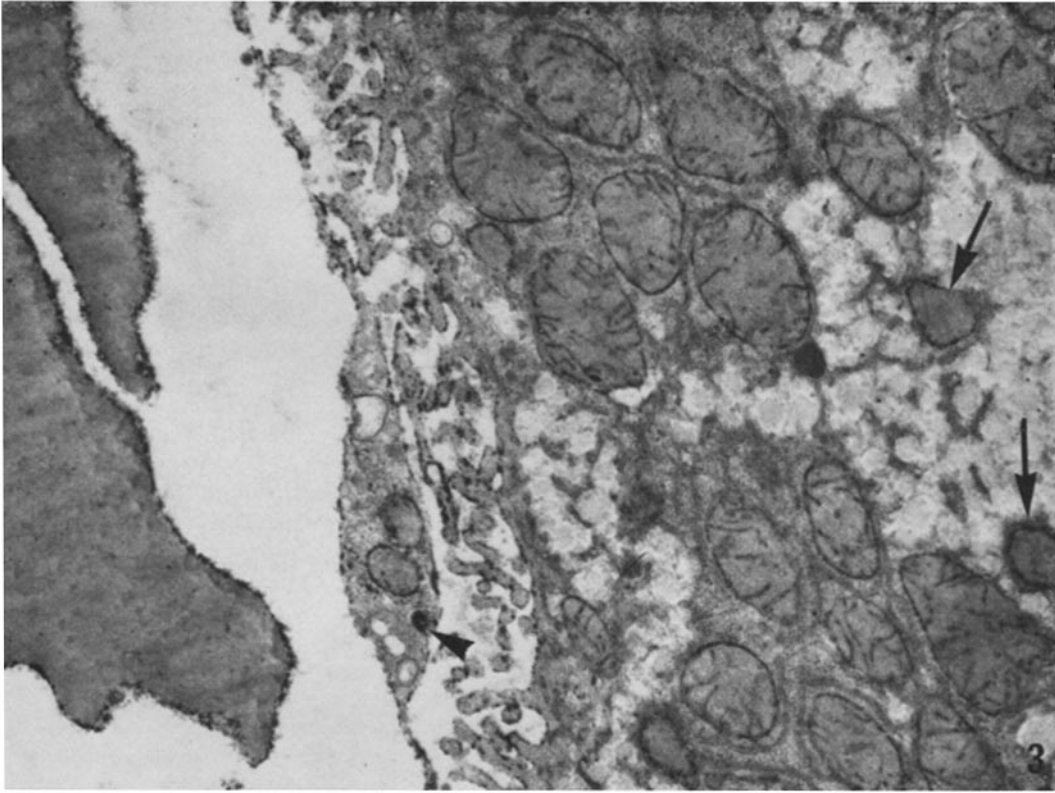
Tissues prepared in the same manner as indicated above but incubated in a medium containing D-aspartic acid instead of L-aspartic acid did not show electron-opaque deposits at any site where enzymatic activity was manifest in the experimental series (Fig. 18). Specimens of liver, muscle, heart, brain, intestine, and testis were included in these control experiments. Generally, the lead contamination in these tissues was remarkably low.

DISCUSSION

There is little doubt that the addition of ketoglutarate to the fixative minimizes enzymatic inactivation and permits a more complete cytochemical demonstration of aspartate aminotransferase activity. These results are in accord with those obtained from model studies of the histochemical techniques used (29). Such correlation calls for further monitoring of cytochemical reactions with model systems similar to that described, in the application of cytochemical methods to ultrastructural use. As expected from the model study, formaldehyde proved less detrimental for enzy-

FIGURE 3 Liver fixed for 12 min in 1% glutaraldehyde with added ketoglutarate. In addition to mitochondria, peroxisomes (arrows), hepatocytic microvilli, micropinocytic vesicles (arrowhead), and the plasma membrane of erythrocytes show evidence of activity. Magnification, 20,000 X.

FIGURE 4 Arteriolar endothelium from striated muscle, fixed for 12 min in 1% glutaraldehyde and ketoglutarate. Enzymatic activity is present in micropinocytic vesicles (arrows) and the junction region (arrowheads). Lead hydroxide. Magnification, 28,000 X.



matic activity than glutaraldehyde, although the latter gives better fixation of ultrastructure. A combination of both fixatives, as used by Lee and Torack (19), and substrate protection may prove ideal in future electron microscopic studies.

The mitochondrial localization of aspartate aminotransferase activity was expected both from a number of biochemical studies (2, 3, 5, 6, 7, 9, 22, 25, 31) and from the more recent ultrastructural evidence in rat liver and heart (18, 19, 20). This type of localization was readily obtained, and did not necessitate substrate protection for its demonstration within the time limits of the fixation periods used.

The available evidence indicates that the mitochondrial isozyme bears a close relationship to the mitochondrial membranes since sonication and detergents largely fail to solubilize the enzyme (1, 5). Recently Schnaitman and Greenawalt (30) have reported that aspartate aminotransferase is intimately related to the inner membrane-matrix fraction of mitochondria. These results are in accord with the finding of reaction product between the inner and outer mitochondrial membranes and extending into the cristae mitochondriales, suggesting a close relationship to the inner mitochondrial membrane. It was noted that, in some hepatocytes, not all mitochondria showed the presence of enzymatic activity. It is not known whether this represents a fixation artifact or if it is an indication of a mitochondrial population within hepatocytes which is relatively poor in aspartate aminotransferase. Light microscopical studies indicate that in mouse liver the periportal hepatocytes possess a greater degree of enzymatic activity than the hepatocytes surrounding the central vein. This may be further evidence of a bimodal distribution of hepatocytic mitochondria. Swick et al. (33) have provided some evidence that this, indeed, may be the case not only for aspartate aminotransferase but for other mitochondrial enzymes as well.

Biochemical studies have not hinted at any

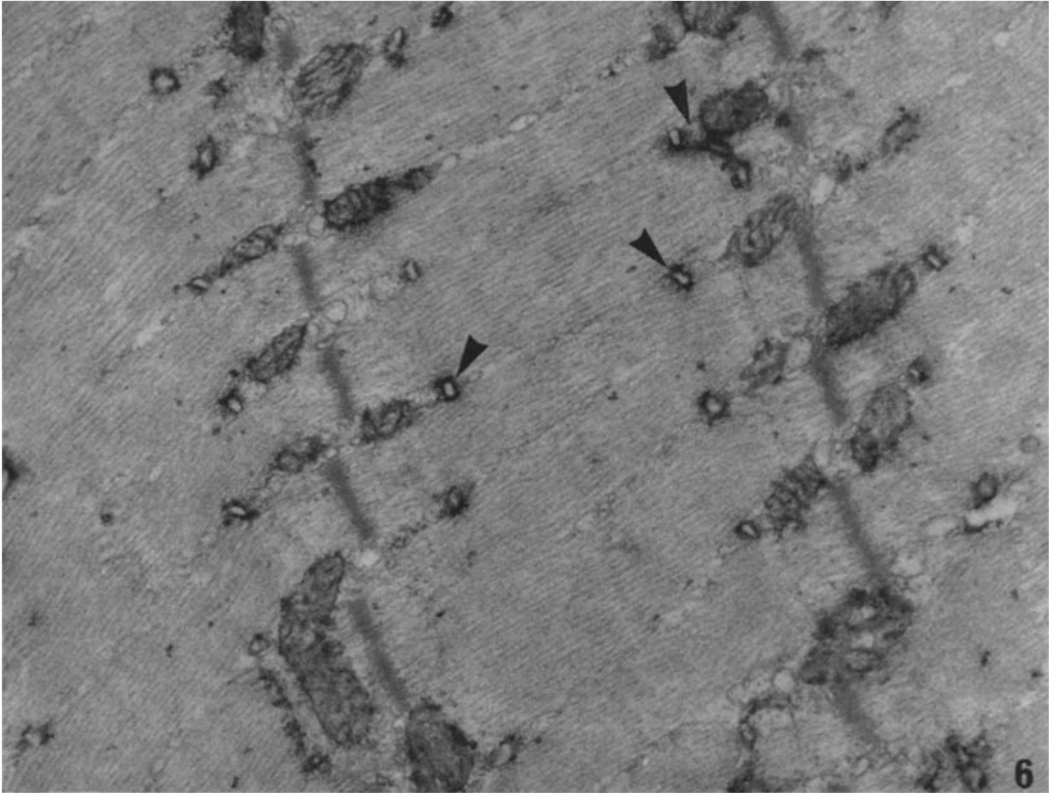
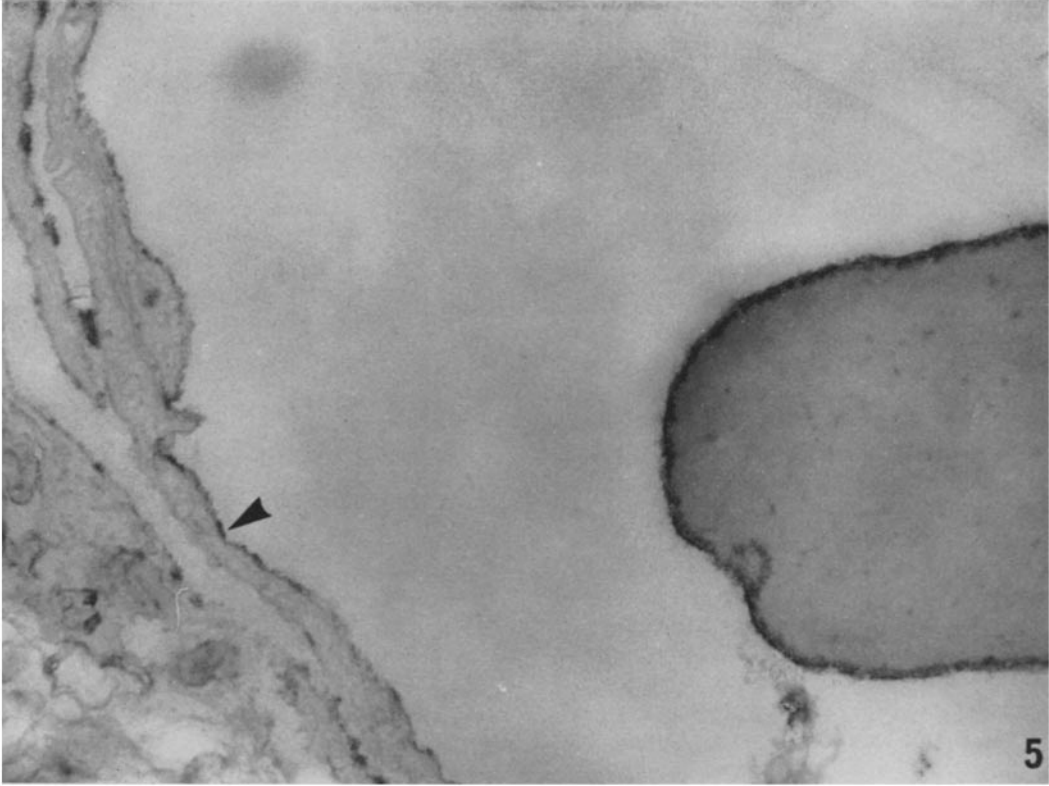
particular localization for the soluble isozyme (2, 3, 5, 6, 7, 9, 22, 24, 25, 30). The term "soluble" merely indicates that upon differential centrifugation the isozyme is found in the supernatant fraction of the cellular homogenate, and it generally does not imply that the isozyme originates in any particular organelle. It was therefore somewhat unexpected that reaction product was present on cytoplasmic structures other than mitochondria, while similar structures in control tissues were not stained. These extramitochondrial sites became the necessary candidates for the localization of soluble isozyme activity *in vivo*. The effect of fixation (by formaldehyde or glutaraldehyde) and substrate protection at these sites was noted since this had already been assessed for the soluble isozyme in experiments utilizing the model system (29). Thus the greater sensitivity of the soluble isozyme to fixatives and the greater beneficial effect of substrate protection on this isozyme were used as a guide to which type of isozyme was being localized. Moreover, the enzymatic activity of erythrocytes, which have been shown to contain only the soluble isozyme (27), paralleled the activity of the soluble isozymes in the model studies (29).

All this indicates that the electron-opaque deposits found in erythrocytes, bacteria, and plasma membranes of other cells are due to the activity of the soluble enzyme. Though there is good evidence that the deposits at these sites are due to the activity of the soluble enzyme, the question whether they demonstrate the exact site of the enzyme as it occurs *in vivo* is more difficult to answer.

During the fixation procedure the enzyme may move from the cytoplasm or from some extracellular space to the membrane location. Whether this will be the case will depend on the velocity of such diffusions, on the rate of the fixation process, and on the firmness with which the enzyme is bound to a certain structure. Independent evidence to exclude enzyme movements prior to fixation is difficult to obtain at the moment. The

FIGURE 5 Cerebral venule fixed in 3.7% formaldehyde and added ketoglutarate, for 12 min. Reaction product is present on the erythrocytic plasma membrane, the endothelial cell membrane (arrowhead), and the membrane of pericytes. Unstained. Magnification, 34,000 X.

FIGURE 6 Striated muscle fixed in 1% glutaraldehyde and added ketoglutarate for 12 min. Reaction product is present in the outer chamber of mitochondria and surrounds transverse tubules (arrowheads). Lead hydroxide. Magnification, 30,000 X.



same holds true for the possibility that the precipitated lead salt of oxaloacetic acid does not precipitate exactly at the site of the enzyme but at some distance from its original site which has some special affinity for lead or oxaloacetic acid.

The fact that the ultrastructural localization of the cationic isozyme is in accord with biochemical findings—which locate this enzyme in the mitochondrial fraction—supports the suggestion that the cytochemical fixation and staining procedure also has some merit in pointing to the cellular localization of the soluble enzyme. The fact that biochemical studies so far have not pointed to any particular localization of the soluble enzyme does not in itself prove that in the *vivo* localization of the enzyme is not in or on membranes. Loss of components originally situated on or in membranes during the preparation procedures now in use cannot be excluded. In the case of sponge cells, it was found that macromolecular products localized at the cell surface could be washed off even by treatment of the cells with cold seawater free of divalent cations (26). Accepting the present cytochemical evidence to represent the true location, it may indicate that the enzyme is associated with the glycocalyx of a number of cell types. Since the enzyme is present in the sera of normal animals (6, 7, 21, 31) one also has to consider whether the enzyme has precipitated onto the cell surface as a consequence of the fixation procedure.

The presence of enzymatic activity within the deeper endothelial micropinocytic vesicles indicates that, in this case, absorption of enzyme as the result of fixation is unlikely. The presence of

enzymatic activity in the interendothelial junctions also indicates that at least part of the activity detected may be inherent to a membranous or glycocalyceal component since the large molecular weight (24) (120,000) of the soluble isozyme would hinder its passage through the junction. Moreover, the presence of activity on the surface of cell types not directly in contact with the vascular space, such as bacteria in the lumen of the gut, favors to some extent a primary plasma membrane localization.

The physiological significance of plasmalemmal localization remains conjectural. Glutamate, and to some degree aspartate, two substances involved in the enzymatic catalysis of aspartate aminotransferase, induce cell membrane depolarization (4, 10, 11, 14, 16, 36, 37). It is not known if the enzyme is involved in the selective transport of amino acids across cell membranes, although glutamate will accumulate in isolated brain tissues against a concentration gradient (32).

Cell membrane-bound soluble isozyme may thus be implicated in glutamate-induced neuronal depolarization (4, 10, 11, 16). Biochemical evidence is available for its presence in the synaptosome fraction of brain (8). Similarly, it may be of importance in the depolarization of striated muscle by glutamate as occurs in insect (14, 36, 37) and crustacean muscle (34). In this respect, its distribution near elements of the transverse tubular system which is involved in the inward spread of depolarization (13), and its resemblance to the distribution of choline esterase (35), are intriguing.

The presence of reaction product on the limiting

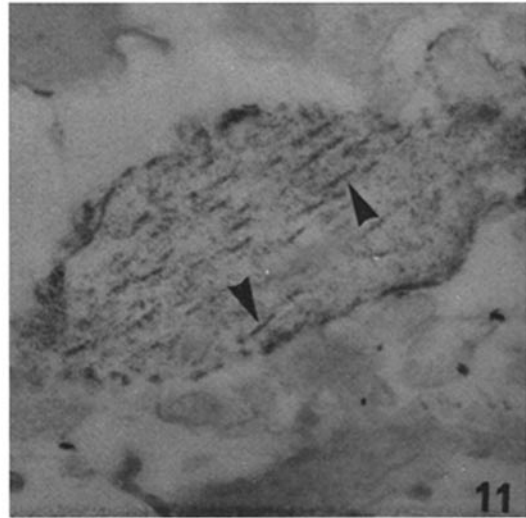
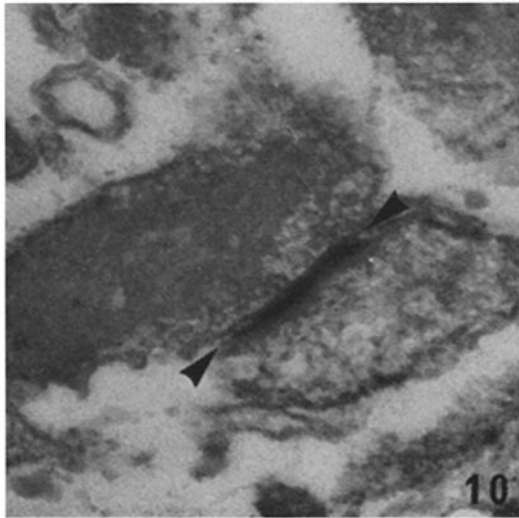
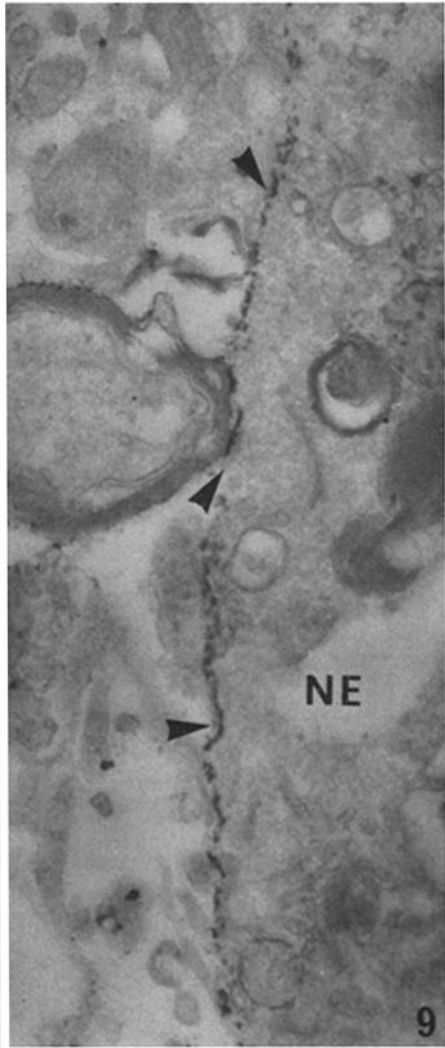
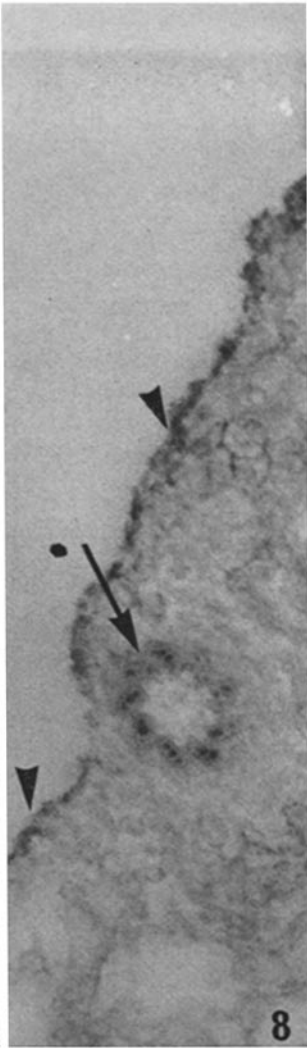
FIGURE 7 Striated muscle fixed in 3.7% formaldehyde with added ketoglutarate for 12 min. Reaction product is present on the cell membrane (upper arrowheads) and on the A-I band junction (lower arrowheads). Unstained. Magnification, 20,500 ×.

FIGURE 8 Fibroblast fixed in 3.7% formaldehyde with added ketoglutarate. Reaction product is present in the plasma membrane (arrowheads) and the component tubules of a subjacent centriole (arrow). Unstained. Magnification, 70,000 ×.

FIGURE 9 Neurone (NE) fixed in 1% glutaraldehyde with added ketoglutarate for 7 min. Reaction product is present on the plasma membrane of the neurone (arrowheads). Unstained. Magnification, 35,000 ×.

FIGURE 10 Mouse brain fixed for 7 min in 1% glutaraldehyde and added ketoglutarate. Reaction product (arrowheads) is present in the gap region of a synapse. Magnification, 60,000 ×.

FIGURE 11 Mouse brain fixed for 7 min in 1% glutaraldehyde and added ketoglutarate. Reaction product is present in the neurotubules of a dendritic process (arrowheads). Unstained. Magnification, 32,000 ×.



membranes of hepatocytic peroxisomes confirms the findings of Lee and Torack (19) in the rat. Enzymatic activity at this site is also more susceptible to the effects of glutaraldehyde fixation than mitochondrial activity, again suggesting that such lead deposits represent sites of activity of the soluble isoenzyme of aspartate aminotransferase. A similar proposition has been made by Lee and Torack (19). They suggest that the discrepancy between these histochemical data and the biochemical findings of Baudhuin et al. (1), who failed to demonstrate aspartate aminotransferase in peroxisomes, may be due to loss of this soluble isozyme during the fractionation procedure.

Generally, it seems that sites of activity of the biochemically defined soluble isozyme are closely related to structural and often membranous components of the cell. Perhaps its physico-chemical relationships to these structures are such that the enzyme molecules are easily detached during the trauma of high speed centrifugation.

Reaction product was detected in only a few centrioles, neurotubules, and axial tubules of the tails of spermatozoa. Negative results may be due to low enzyme concentrations, with subsequent failure of formation of a reaction product (12). The presence of enzymatic activity on all these structures and on cell membranes points perhaps to an underlying fundamental similarity. A unifying hypothesis based on biochemical and morphological similarities has been proposed by Mazia (23), in an attempt to link these diverse structures. The only situation in which enzymatic activity was detected in the ground substance is in the intestinal goblet cells, and, even there, it appeared in close apposition to cisternae of endoplasmic

reticulum. Since glutamate plays a role in the amination of amino sugars in the early stages of mucopolysaccharide synthesis (15), aspartate aminotransferase may be required in increased amounts in goblet cells which are active in the production of these materials.

These data suggest that the soluble isozyme of aspartate aminotransferase may be involved in many basic cellular mechanisms and reactions which as yet remain partly or completely unsolved. Further information regarding its biochemical and physiological role should prove rewarding.

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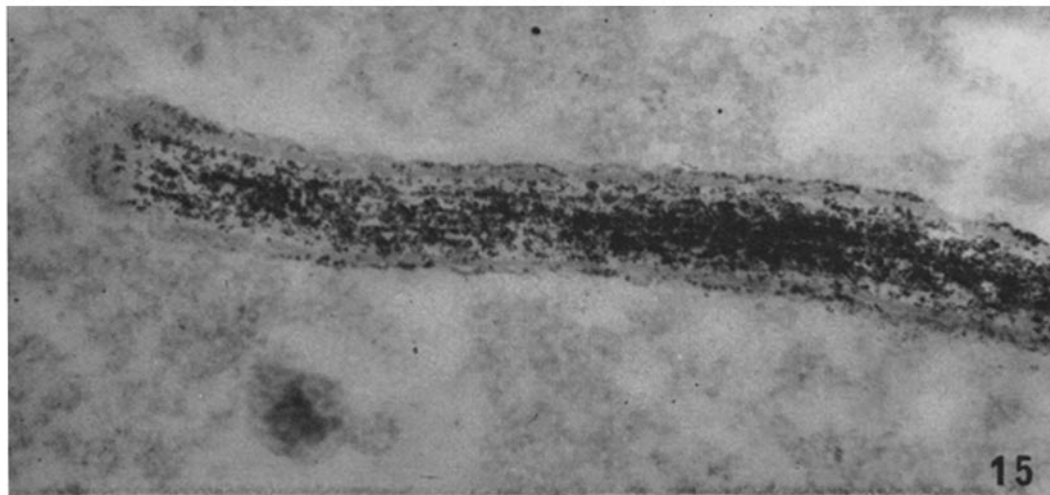
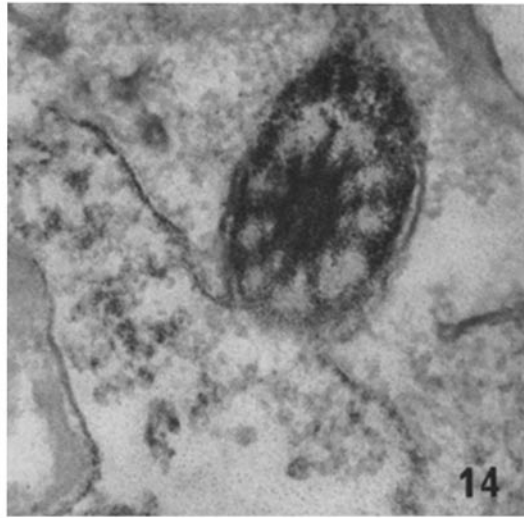
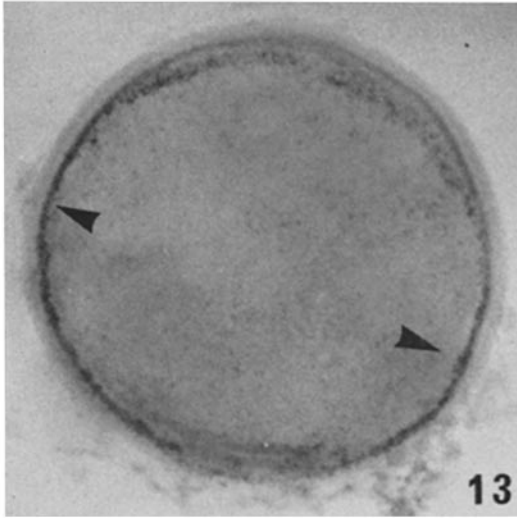
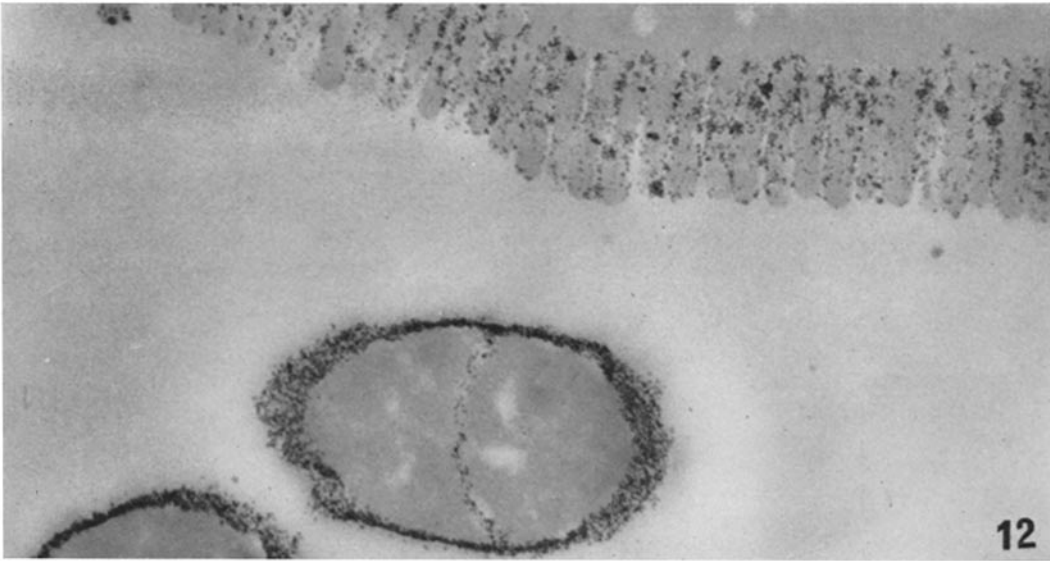
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FIGURE 12 Mouse intestine fixed in 3.7% formaldehyde for 12 min with added ketoglutarate. Reaction product is seen on the microvilli of the columnar epithelial cells and surrounding bacterial cells in the intestinal lumen. Unstained. Magnification, 37,000 X.

FIGURE 13 Bacterial cell in intestinal lumen fixed for 12 min in 3.7% formaldehyde with added ketoglutarate. Reaction product is confined to the cell membrane (arrowheads) and not the capsule of the organism. Unstained. Magnification, 55,000 X.

FIGURE 14 Mouse testis fixed for 12 min in 3.7% formaldehyde with added ketoglutarate. Reaction product is present on the cell membrane and a centriole of a developing spermatid. Unstained. Magnification, 75,000 X.

FIGURE 15 Mouse testis fixed for 12 min in 3.7% formaldehyde with added ketoglutarate. Reaction product is present on the cell membrane and the axial microtubules of spermatozoan tail. Unstained. Magnification, 52,000 X.

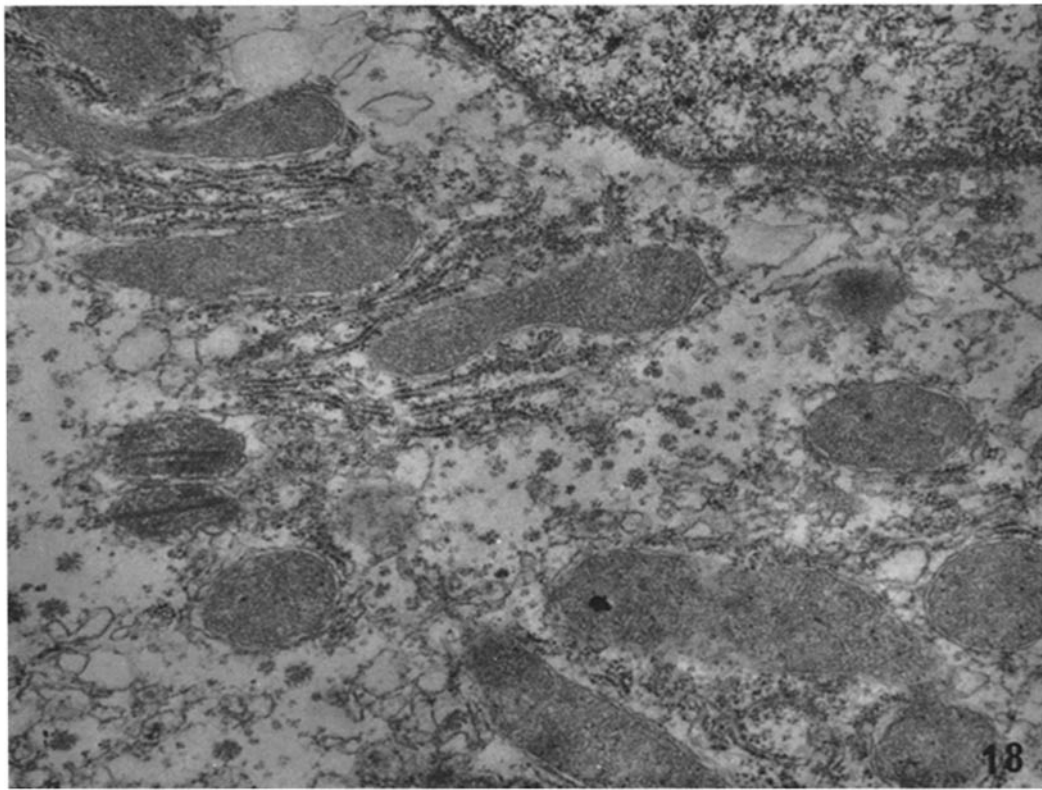
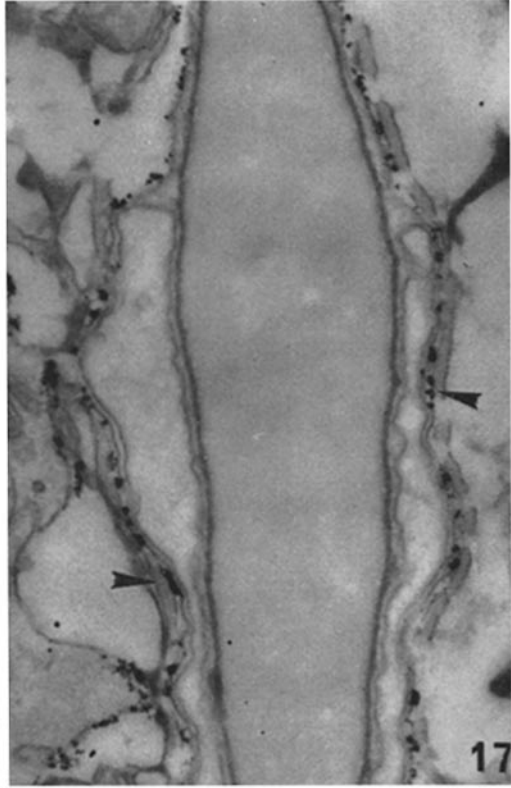
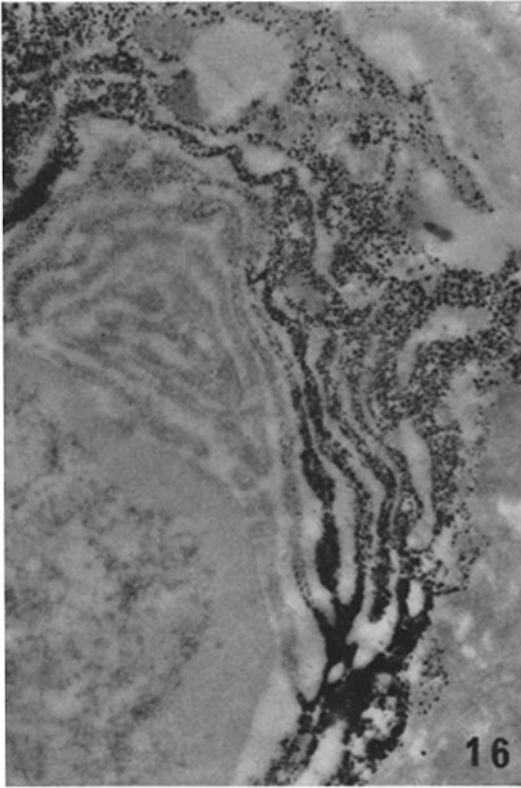


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FIGURE 16 Intestinal goblet cell fixed in 3.7% formaldehyde with added ketoglutarate. Reaction product is present in the ground substance between cisternae of endoplasmic reticulum. Unstained. Magnification, 14,500 X.

FIGURE 17 Mouse testis fixed in 3.7% formaldehyde with added ketoglutarate. Reaction product is found on the plasma membrane of a developing spermatid and on that of an adjacent sertoli cell (arrowheads). Magnification, 36,000 X.

FIGURE 18 Mouse liver fixed for 12 min in 1% glutaraldehyde with added ketoglutarate and incubated in a medium containing D-aspartic acid. Reaction product cannot be found at any site. Lead hydroxide. Magnification, 24,000 X.



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