

# ELECTRON MICROSCOPE STUDIES OF GLUTAMIC OXALACETIC TRANSAMINASE IN RAT LIVER CELL

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## ABSTRACT

Liver tissue of the rat, fixed in glutaraldehyde and formaldehyde, was incubated in a medium which consisted of 20 mM L-aspartic acid, 2 mM  $\alpha$ -ketoglutaric acid, 50 mM imidazole and 6 mM lead nitrate at pH 7.2-7.4. The electron-opaque precipitates, due to glutamic oxalacetic transaminase activity in liver cells, were found to be localized to the cristae and surface membranes of the mitochondria, the limiting membrane of the microbodies, and the nuclear membrane. Sucrose storage and trauma resulted in altered morphology and diminished final product intensity in mitochondria, but the microbody enzyme activity disappeared completely under these conditions. These distinctive responses of enzymatic activity are considered to indicate a difference in either the enzyme protein or its membrane attachment to these two sites. The use of a buffered dehydrating ethanol series to prepare tissue blocks for electron microscopy appeared to result in more precise intracellular localization of enzymatic reaction product.

## INTRODUCTION

The importance of glutamic oxalacetic transaminase (GOT) in amino acid metabolism has stimulated much interest in its subcellular localization, particularly in those organs possessing high levels of this enzyme such as heart, skeletal muscle, kidney, and liver (1). While the mitochondrial GOT is believed to account for most of the enzyme activity in the tissue homogenates, considerable variation exists in the reports of its distribution among individual subcellular fractions (2, 4, 6, 11). The present communication deals with the use of an electron histochemical technique to study the localization of GOT activity in intact liver cells, based on the precipitation of the lead salt of oxalacetic acid (8), a primary reaction product of transamination.

## MATERIALS AND METHODS

### *Fixation Procedure*

Young adult Wistar rats weighing 150-250 g were perfused with 200 ml of cold (4°C) fixative which consisted of 1% glutaraldehyde (Biological Grade, Fisher Scientific Company, Fair Lawn, N.J.) and 3.7% formaldehyde (Amend Drug and Chemical Co., Inc., New York.) in 0.05 M imidazole-nitric acid buffer at pH 7.2 and containing 0.3 M sucrose. The perfusate was introduced under high pressure into the thoracic descending aorta of a lightly ether-anesthetized animal, with the aorta proximal to this point clamped. The entire perfusion procedure was completed within 2-3 min. A small piece of the perfused liver was excised and minced rapidly into small blocks no more than 1 mm<sup>3</sup> in size. These tissue

blocks were fixed for another 30 min in cold (4°C) 3.7% formaldehyde in 0.05 M imidazole-nitric acid buffer containing 0.3 M sucrose, and were subsequently washed in imidazole-buffered 0.3 M sucrose for 1 hr with four changes of wash medium.

#### *Enzymatic Incubation*

The fixed and washed tissue blocks were incubated at room temperature, 25° + 3°C, for 15–30 min in a modified GOT medium which contained 20 mM L-aspartic acid, 2 mM  $\alpha$ -ketoglutaric acid, 50 mM imidazole, 6 mM lead nitrate, and 0.3 M sucrose, pH 7.2–7.4, made up in a manner described previously (8). Cryostat sections cut 10  $\mu$  thick from the perfused liver and postfixed in 3.7% formaldehyde for 30 min were also incubated in this medium, treated with ammonium sulfide, and mounted on glass slides in glycerogel (9). Tissue blocks of similar rat liver were also incubated for 30 min at room temperature in the following control media:

Control Medium A: 20 mM L-aspartic acid, 6 mM lead nitrate, 50 mM imidazole in 0.3 M sucrose, pH 7.2–7.4.

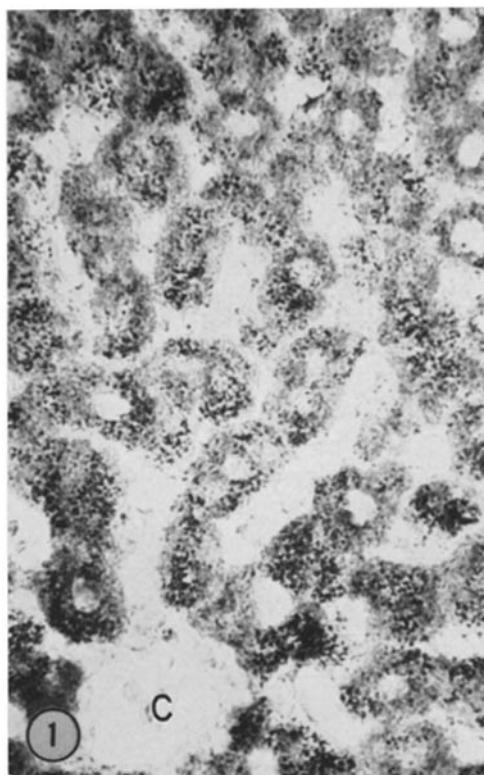


FIGURE 1 Light micrograph of part of a hepatic lobule, showing cytoplasmic granular reaction product of GOT activity. Central vein (C).  $\times 750$ .

Control Medium B: 20 mM D-aspartic acid, 2 mM  $\alpha$ -ketoglutaric acid, 6 mM lead nitrate, 50 mM imidazole in 0.3 M sucrose, pH 7.2–7.4.

#### *Tissue Preparation for Electron Microscopy*

The incubated tissue blocks were washed for 5 min in 20 mM L-aspartic acid in 0.3 M sucrose buffered by 0.05 M imidazole at pH 7.3, postfixed in 1%  $O_3O_4$  buffered with Veronal to pH 7.2–7.4, rinsed in 0.05 M imidazole-nitric acid buffer at pH 7.3, and dehydrated in an ethanol series which was made up by diluting absolute alcohol with 0.05 M imidazole-nitric acid buffer, pH 7.5. Some of the incubated tissue blocks were dehydrated in the routine ethanol series which was made up by diluting absolute alcohol with distilled water instead of imidazole-nitric acid buffer. All tissue blocks were embedded in Epon, sectioned with an LKB ultratome, and examined with an RCA EMU 3-G electron microscope. Some thin sections were stained with lead citrate.

#### *Effect of Sucrose Storage on GOT Activity*

A piece of fresh liver taken from an anesthetized rat was minced rapidly in cold 0.25 M and 0.3 M sucrose and stored in the same solution at 4°C. After 5 min, 20 min, 1 hr, and 2 hr respectively, small tissue blocks were removed and fixed in a mixture of 1% glutaraldehyde and 3.7% formaldehyde for 2 min, followed by an additional fixation in 3.7% formaldehyde for another 30 min. Another small piece of fresh rat liver was minced in the glutaraldehyde-formaldehyde mixture immediately after removal from the same animal and fixed in an identical manner. These fixatives all contained 0.3 M sucrose and 0.05 M imidazole-nitric acid as mentioned above. All the fixed tissue blocks were then incubated for GOT activity, postfixed in osmium tetroxide and embedded in Epon exactly as in the previous experiment.

## RESULTS

#### *Light Microscopy*

The final reaction product was present as distinct fine particles in the cytoplasm of the hepatocyte, and was similar to that in the cryostat sections fixed by immersion method (Fig. 1). There were no obvious deposits in the nuclei. Very little reaction product was noted in the Kupffer cells.

#### *Electron Microscopy*

Examination of the perfused tissue revealed the fine structure of the hepatocyte to be well preserved. In particular, the mitochondria had a

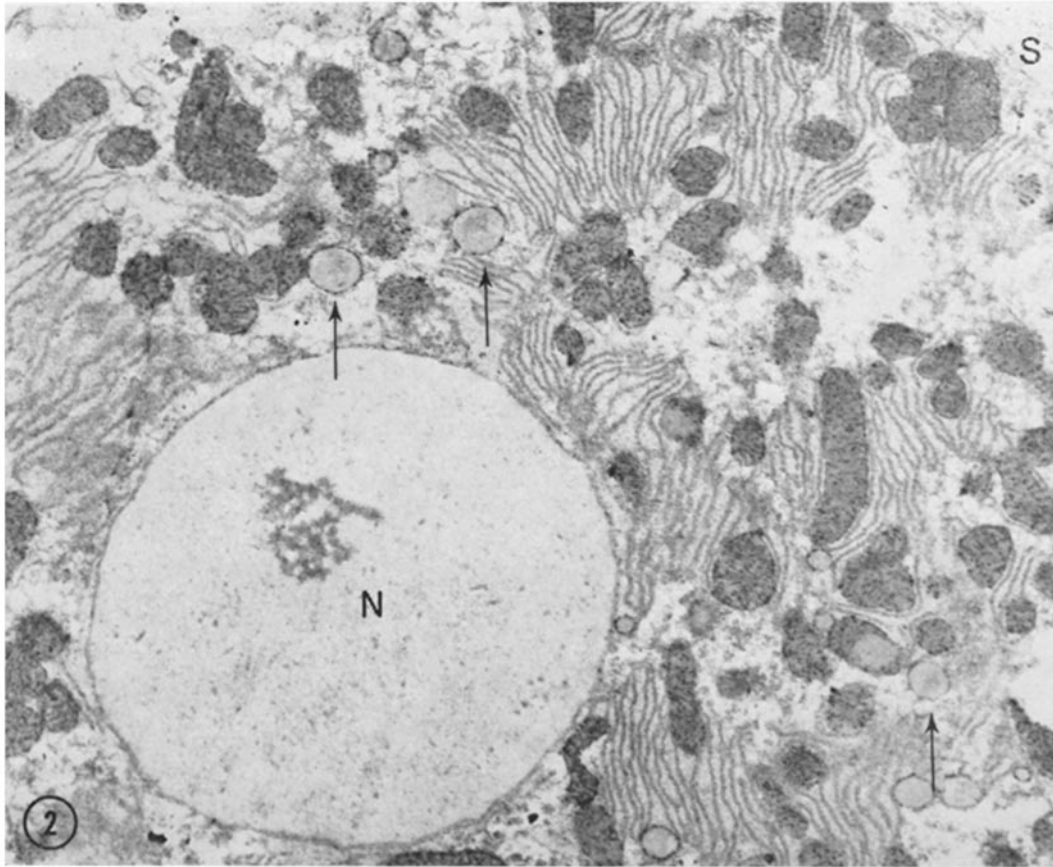


FIGURE 2 Electron micrograph of a liver cell. The tissue was perfused, fixed, incubated in GOT medium for 20 min and dehydrated in buffered ethanol series. The thin section was stained with lead citrate. The final reaction product is deposited in the mitochondria, at the surface of the microbodies (arrows), and in the nuclear membrane. No deposits are present in the nuclear substance (*N*). Space of Disse (*S*).  $\times 11,000$ .

compact appearance. The electron-opaque precipitates were discretely localized to the surface membranes and the cristae of the mitochondria, the limiting membranes of the microbodies, and the nuclear membranes of the liver cells (Figs. 2-5). Within the mitochondria the final product was found in the spaces between the limiting membranes and between the cristate membranes or directly on the surfaces of these membranes. No reaction product was observed in the central portion of the microbodies even after a 1 hr incubation. The nucleoplasm, endoplasmic reticulum, and the Golgi apparatus were unstained after a 20 min incubation. After 1 hr, an irregular light deposit was occasionally seen in all these

structures, but these findings were considered to be of questionable specificity. The use of an unbuffered ethanol series more often resulted in a nonspecific deposition of lead, particularly in the nucleus and in the endoplasmic reticulum (Fig. 6).

Examination of the tissue blocks incubated in the control media revealed no lead deposits in the thin sections (Fig. 7).

After storage in sucrose for 5 min, the cells at the periphery of the fresh tissue blocks appeared to be seriously traumatized with ruptured cell membranes. In these cells the mitochondria were considerably enlarged and the cristae were focally irregularly dilated. These changes were not observed at the periphery of blocks from perfused

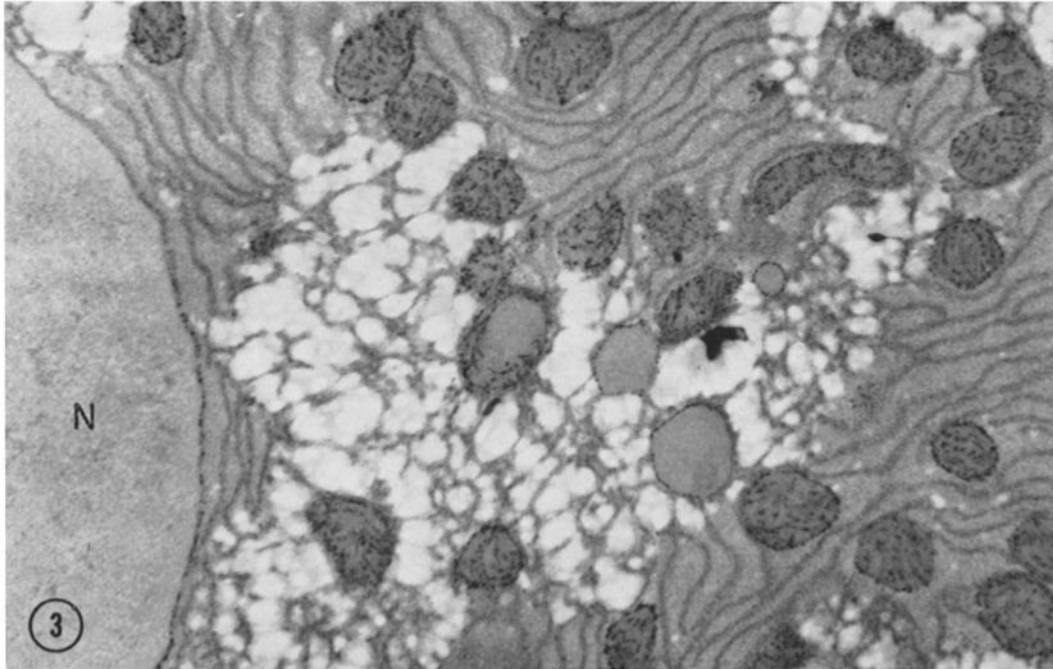


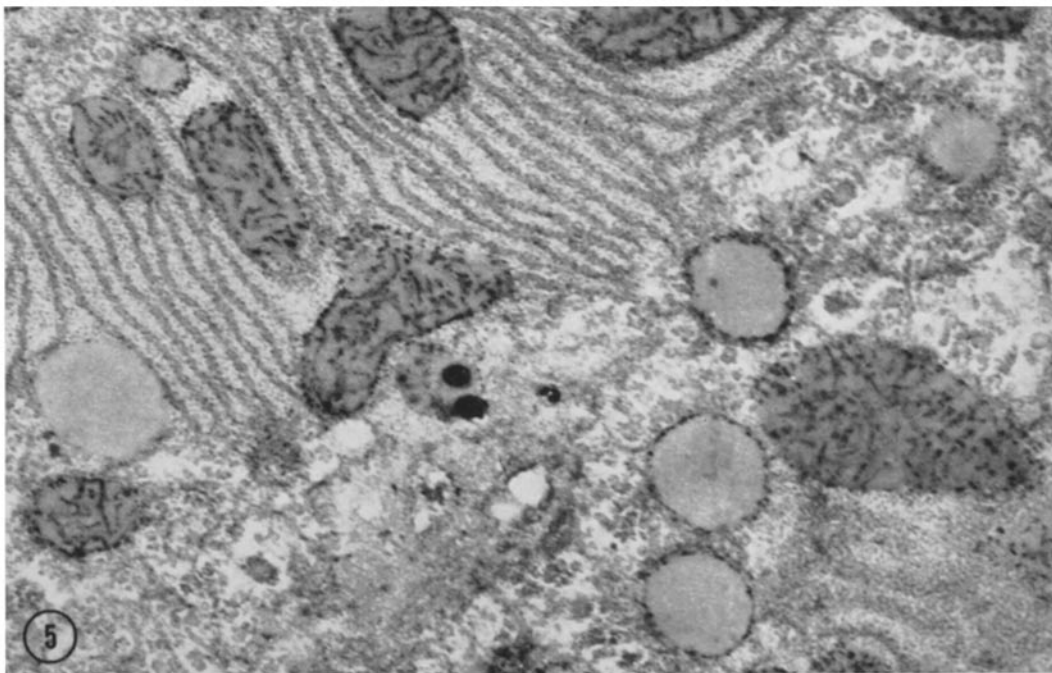
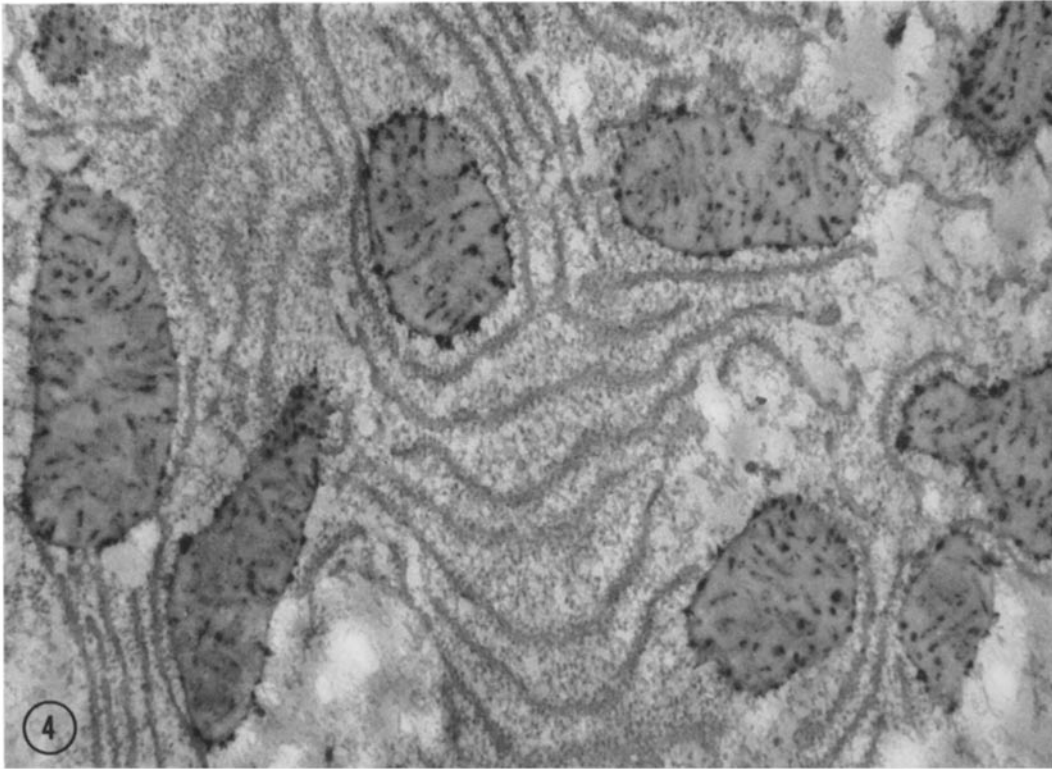
FIGURE 3 Same protocol as in Fig. 2 except that the thin section was unstained. The reaction product is at the surface membranes and in the cristae of the mitochondria, at the surface of the microbodies, and in the cisternae of the nuclear membrane. No deposits are present in the nuclear substance (*N*).  $\times 20,000$ .

liver. The final enzymatic reaction product in the altered mitochondria was reduced markedly as compared with the slender mitochondria in the adjacent intact cells which still contained prominent precipitates (Fig. 8). The remaining precipitates in the altered mitochondria appeared as fine granules within the matrix between these empty cristae. No GOT activity was demonstrated on the surface of the microbodies of the ruptured cells (Fig. 9), and only traces of activity were present at the surface of the microbodies in the intact cells (Fig. 8). When the duration of sucrose storage was prolonged to 20 min or more, most of the microbodies lost their GOT activity entirely even in the intact cells. In these cells, the enzyme activity in many mitochondria was reduced, but it was still demonstrable by histochemical means (Fig. 10). Thin sections of the tissue blocks which were fixed in the glutaraldehyde-formaldehyde mixture immediately after removal contained a reaction product in mitochondria, microbodies, and nuclear membrane, exactly like that in the sections of liver cells fixed by perfusion.

#### DISCUSSION

Electron microscope study of GOT activity in liver cells has revealed the final product to be localized to mitochondria, microbodies, and the nuclear envelope. The prominent intramitochondrial location of the enzymatic reaction product is in excellent agreement with the biochemical data which have revealed most activity to be in the mitochondrial fraction following differential centrifugation of homogenized tissue (2, 4, 6, 11). These previous experiments have indicated a close relationship of the mitochondrial GOT to membranous structure, since sonication and the use of detergents have largely failed to solubilize the enzyme (2, 4). The localization of the final product within the limiting membranes and cristae of mitochondria also suggests an intimate association of this enzyme with membranes.

The presence of GOT activity at the surface of the microbodies has not been supported by fractionation studies (3). This discrepancy between the biochemical and histochemical data may be due to an activation of a latent enzyme at



**FIGURE 4** A higher magnification of reacted mitochondria, in which the final enzymatic reaction product is either on or between the surface membranes and the cristate membranes.  $\times 37,000$ .

**FIGURE 5** An electron micrograph of a liver cell showing reaction product on the surface of four microbodies.  $\times 29,000$ .

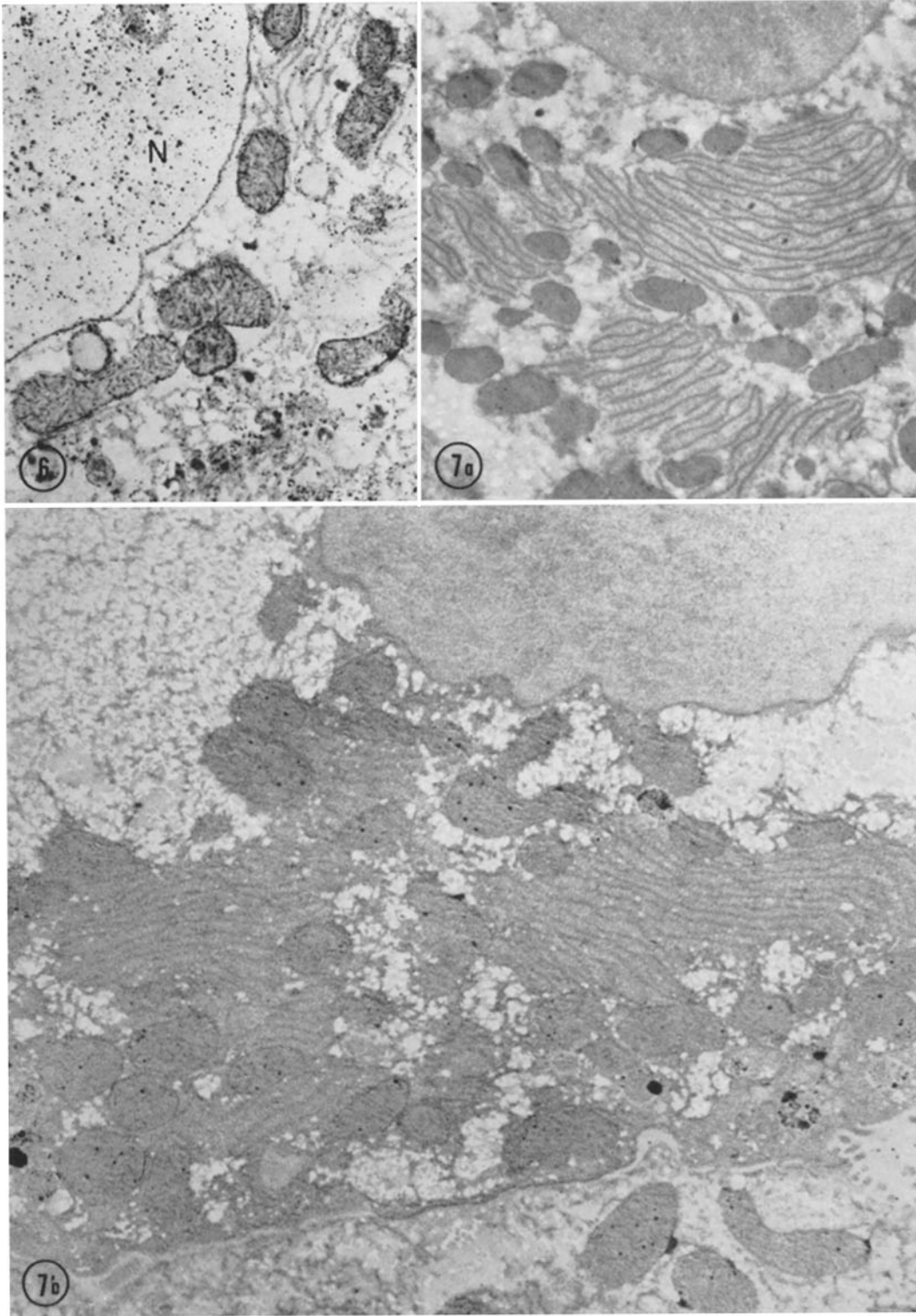


FIGURE 6 Same protocol as in Fig. 2 except that the incubated tissue blocks were dehydrated in unbuffered ethanol series. The section was unstained. Nonspecific lead deposits are noted in the nuclear substance (*N*) and in the cytoplasm.  $\times 22,000$ .

FIGURE 7a No lead deposits are noted in the cells of the tissue blocks incubated in a medium lacking  $\alpha$ -ketoglutaric acid for 30 min. Section was unstained.  $\times 8,000$ .

FIGURE 7b Similar negative results were obtained when the *L*-aspartic acid of the medium was replaced by equimolar *D*-aspartic acid. Section was unstained.  $\times 17,000$ .

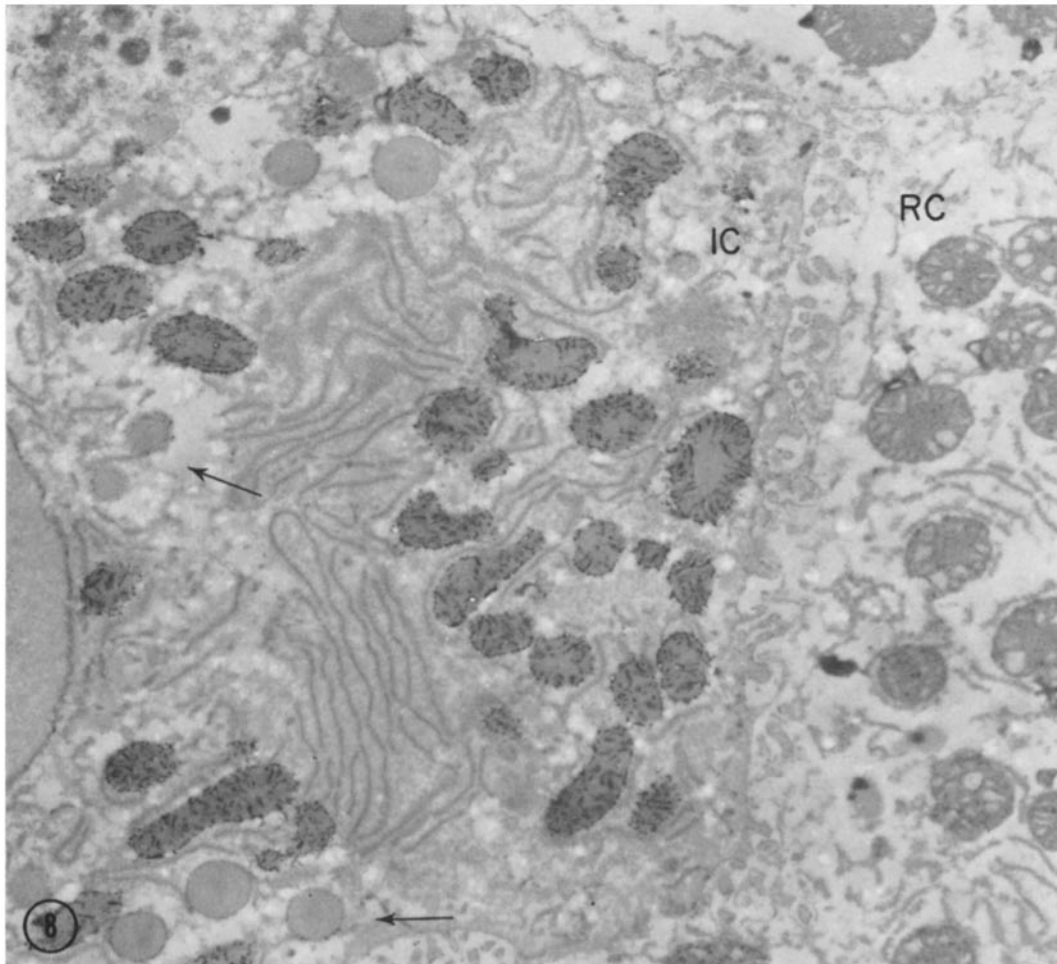


FIGURE 8 Parts of an apparently intact cell (*IC*) and a ruptured cell (*RC*) at the surface of a tissue block after a 5 min storage in cold isotonic sucrose. After fixation and incubation for GOT activity, there is a considerable amount of reaction product in the mitochondria of the intact cell whereas only traces of reaction product are demonstrated at the surface of the microbodies (arrows) in the same cell. Compared with those in the intact cell, the mitochondria of the ruptured cell are studded with many empty dilated cristae and the GOT reaction product is markedly reduced and dispersed in the matrices. The section was unstained.  $\times 15,000$ .

the microbody surface by the histochemical method or to a dissociation of the microbody surface enzyme during the fractionation procedure. An activation of GOT by a low concentration of lead ion has been previously reported (10). On the other hand, no reaction product is visible at the surface of microbodies after a 5- to 20-min storage in cold isotonic sucrose, and this enzyme activity does not appear to be as firmly bound to membranes as the mitochondrial enzyme which re-

mains active. Therefore, the lack of GOT activity in the purified microbody fraction which is obtained by ultracentrifugation for 3 hr (3) might be a result of solubilization. The supernatant in the fractionation procedure always contains considerable activity, which could be partly derived from dissociated enzyme. Furthermore, two isozymes with distinct electrophoretic properties are known (2, 5) and the anionic intracellular GOT is thought to be more soluble. The elevation

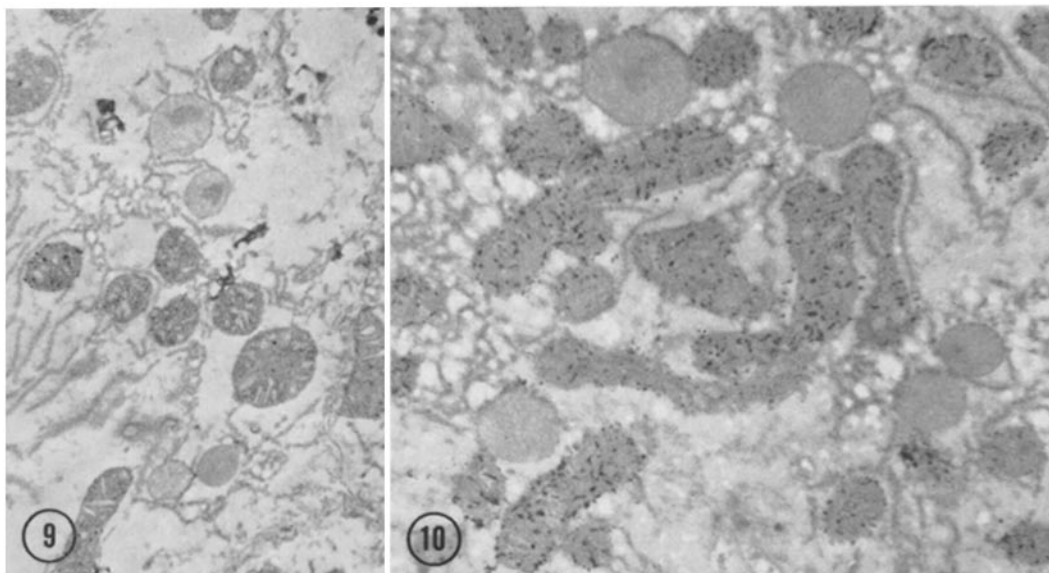


FIGURE 9 Part of a ruptured liver cell at the surface of a tissue block after a 5 min storage in cold isotonic sucrose. After fixation and incubation for GOT activity, only a small amount of reaction product is shown as fine granules in the matrices of the mitochondria as in Fig. 8. No reaction product is noted at the surface of the microbodies. The section was unstained.  $\times 12,000$ .

FIGURE 10 Part of an intact cell near the surface of the block after a 20 min storage in cold isotonic sucrose. After fixation and incubation for GOT activity, no reaction product is formed at the surface of the microbodies whereas occasional dense deposits are still localized in the dilated mitochondrial cristae. The section was unstained.  $\times 22,000$ .

of serum GOT activity in disease is considered to be due to a cellular release of this anionic enzyme (5). The possibly more soluble microbody enzyme could contribute to this elevation; however, these studies neither prove nor disprove this possibility.

The nuclear membrane has been considered to be a specialized element of the endoplasmic reticulum (13). However, the present study shows that GOT activity is consistently demonstrated in the cisternae of the nuclear membrane in the absence of discernible activity in the endoplasmic reticulum of the same cell. This may imply that a biochemical difference could exist between these two subcellular structures although they are morphologically continuous.

There are no precipitates in the electron micrographs of the control experiments. Accordingly, a possible interference by amino acid oxidase or keto-acid decarboxylase which may attack L-aspartic acid or  $\alpha$ -ketoglutaric acid individually appears unlikely. The importance of maintaining a slightly alkaline pH in the dehydrating ethanol

series is illustrated by the nonspecific deposition of lead both in the cytoplasm and in the nuclear substance when unbuffered alcohol is used for dehydration. A similar alkalinity of the post-incubation washing medium and the osmium tetroxide postfixation solution is considered just as important, even though the effects of acidity in these solutions has not been separately studied. An acid pH may have several undesirable effects on the localization of reaction product. First, a cation-accelerated decarboxylation of oxalacetate has been shown to occur at or below pH 6.9 (7, 10, 12). This would result in the formation of lead pyruvate, which is more soluble than oxalacetate, and carbon dioxide which could be subsequently precipitated as lead carbonate. Secondly, lead oxalacetate is soluble below pH 6.0 and, as this pH is approached, a mobilization of these precipitates could occur. Finally, acidic pH has been known to predispose to non-enzymatic metallic precipitates in tissue, particularly of silver and lead. We also feel that the presence of



aspartate in the postincubation washing medium helps to reduce nonspecific lead precipitates by chelating lead ion.

The effect of sucrose storage, particularly upon mitochondria, is of interest since both the morphology and the enzyme activity appear to be altered by this procedure. This distortion of mitochondrial structure and concomitant loss of GOT activity appear to result from trauma as well as from sucrose storage. This decrease in enzyme activity could possibly be due to a dissociation of enzyme from the mitochondrial membranes or due to an alteration of the physicochemical state of the membrane itself, with the enzyme being more readily exposed to the destructive effect of the fixatives. The mitochondrial

GOT has been shown to be separated with difficulty, from membranes whereas membrane permeability is readily altered by trauma or anoxia. In addition, purified enzyme has been observed to be readily inactivated by these fixatives (10). Therefore, an inactivation of the enzyme *in situ* by the aldehyde fixative is probably of more importance. This effect of fixation on the GOT activity of mitochondrial fractions obtained by differential ultracentrifugation will be reported separately.

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