

A BIOCHEMICAL AND HISTOCHEMICAL STUDY OF GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITY OF RAT HEPATIC MITOCHONDRIA FIXED *IN SITU* AND *IN VITRO*

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ABSTRACT

Rat liver perfused *in situ* briefly with a glutaraldehyde-formaldehyde mixture was homogenized in isotonic sucrose. The mitochondria, isolated from a homogenate of the perfused liver by differential centrifugation, assumed a slender and compact appearance similar to those often seen in an intact cell. The glutamic oxalacetic transaminase (GOT) activity of this mitochondrial fraction survived an additional formaldehyde fixation and was studied by biochemical and histochemical methods. The biochemical assay of the enzyme activity revealed that the activity was only slightly less than that of an unfixated mitochondrial fraction. The reaction product due to mitochondrial GOT activity was found to be localized to the cristae, as had been demonstrated in an intact liver cell. GOT activity of the mitochondrial fraction isolated from fresh liver tissue homogenate in 0.25 M sucrose was inactivated readily by either glutaraldehyde or formaldehyde and was no longer demonstrable by biochemical and histochemical methods after fixation.

INTRODUCTION

The histochemical demonstration of glutamic oxalacetic transaminase (GOT) activity in tissues has revealed physicochemical characteristics different from those of purified GOT, which is more readily inactivated by aldehyde fixation (11, 12). After cold isotonic sucrose storage and traumatic injury of rat hepatocytes the mitochondria appear enlarged and after fixation their GOT activity shows a histochemical reduction (13). These apparent differences in the behavior of the enzyme protein might be related to the action of the fixative and the heavy metal which is used as a capturing reagent in the histochemical method, or to the homogenization and prolonged sucrose storage which is required to

obtain a mitochondrial fraction. The use of a mitochondrial fraction for histochemical demonstration of enzyme activity offers an opportunity to compare a quantitative assay and a qualitative assay of this enzyme activity and hopefully to elaborate the mechanisms by which the enzyme activity is altered during the histochemical or biochemical procedures.

MATERIALS AND METHODS

Effect of Fixative on GOT Activity of Mitochondria Isolated in Sucrose

Young adult male albino rats of the Wistar strain weighing 250 g were used in all these experiments.

The liver tissue from lightly ether-anesthetized animals was homogenized for two min in enough cold 0.25 M sucrose to make a 5% tissue homogenate. After removal of the nuclei and the intact cells, a pellet of mitochondria was isolated from 30 ml of homogenate according to the method of Hogeboom (8). The pellet was resuspended in an original volume of sucrose solution, equally distributed in three test tubes, and centrifuged at 24,000 *g* for 10 min. The supernatant was discarded. 2 ml of 1% glutaraldehyde (Biological Grade, Fisher Scientific Company, Fair Lawn, N.J.) in imidazole-buffered 0.25 M sucrose were added to the first test tube, and the pellet was redispersed. Exactly 2 min later, 20 ml of buffered sucrose was added in order to reduce the concentration of the fixative. The fixed mitochondria were washed three times and resuspended in 10 ml of sucrose. To the second test tube 2 ml of 3.7% formaldehyde (Amend Drug and Chemical Co., Inc., New York.) in buffered sucrose were added. The pellet of mitochondria was redispersed, fixed for 30 min, then similarly washed and resuspended. The mitochondria in the third test tube were washed with sucrose and resuspended as control.

Assays of GOT activity in the mitochondrial suspensions were carried out with the ultraviolet method of Karmen (9). When lead ion was incorporated in the substrate, the colorimetric method of Reitman and Frankel (18) was followed. For these latter determinations, the medium consisted of 100 mM L-aspartic acid, 2 mM α -ketoglutaric acid, 50 mM imidazole and 6 mM lead nitrate. All chemicals and the calibration standard solution for the colorimetric method were obtained from the Sigma Chemical Co., St. Louis, Mo.

Aliquots of the above mitochondrial suspensions were recentrifuged. Some of the mitochondrial pellets thus obtained were incubated in the histochemical GOT medium, postfixed in osmium tetroxide, dehydrated, and embedded in Epon for electron microscope study exactly as the tissue blocks which were previously described (13). Some fragments of pellets were fixed in osmium tetroxide directly for morphologic observation.

Effect of Fixative on GOT Activity of Mitochondria Isolated in PVP-Sucrose

The procedure of the previous experiment was followed except that the sucrose solution used for tissue homogenization and dilution of the fixatives contained 7.3% polyvinylpyrrolidone (16).

Effect of Fixative on GOT Activity of Isolated Mitochondria in the Presence of ATP

A pellet of mitochondria isolated from 5% liver tissue homogenate in 0.25 M sucrose was redispersed in a solution which consisted of 0.125 M KCl, 0.01 M ATP, and 0.02 Tris-HCl, pH 7.4 (14). The suspension was equally divided into three parts. Ten minutes later, concentrated glutaraldehyde and formaldehyde were added to the first and second parts to a final concentration of 1% glutaraldehyde and 3.7% formaldehyde, respectively. The duration of fixation with glutaraldehyde was 2 min, and that with formaldehyde was 30 min. Thereafter, the mitochondria were washed three times and resuspended in the original volume of isotonic sucrose. The third part was similarly washed and resuspended as the control. Assays of GOT activity and preparation of pellets for electron microscope observation were carried out as mentioned above.

GOT Activity of the Mitochondria Fixed In Situ by Perfusion Method

A lightly ether-anesthetized rat was perfused with a mixture of 1% glutaraldehyde and 3.7% formaldehyde in buffered isotonic sucrose for 2 min as previously described (13). Part of the perfused liver was removed, sliced rapidly at a thickness of about 1 mm, and blotted with filter paper. After the excess fixative was removed, a 5% homogenate of the perfused liver tissue was made in imidazole-buffered 0.25 M sucrose, pH 7.2-7.4. The perfused tissue was homogenized for 6 min instead of the 2 min which was used for fresh liver. The pellet of mitochondria isolated from 20 ml of homogenate was equally divided into two parts. Part I was redispersed and fixed in cold 3.7% formaldehyde in buffered sucrose for another 30 min and washed three times with isotonic sucrose. Part II was washed three times without additional fixation. After being resuspended in the original volume of sucrose, assays of GOT activity in both parts were carried out as mentioned above. Fragments of the pellet reformed from Part I suspension were processed for electron microscope studies with and without incubation for GOT activity. In order to biochemically assay the final enzymatic reaction product, another portion of the same pellet was incubated in GOT medium, washed, resuspended in imidazole-nitric acid buffer, and treated with 2,4-dinitrophenyl hydrazine in HCl; the absorption spectrums of the hydrazones were measured and recorded as previously reported (10).

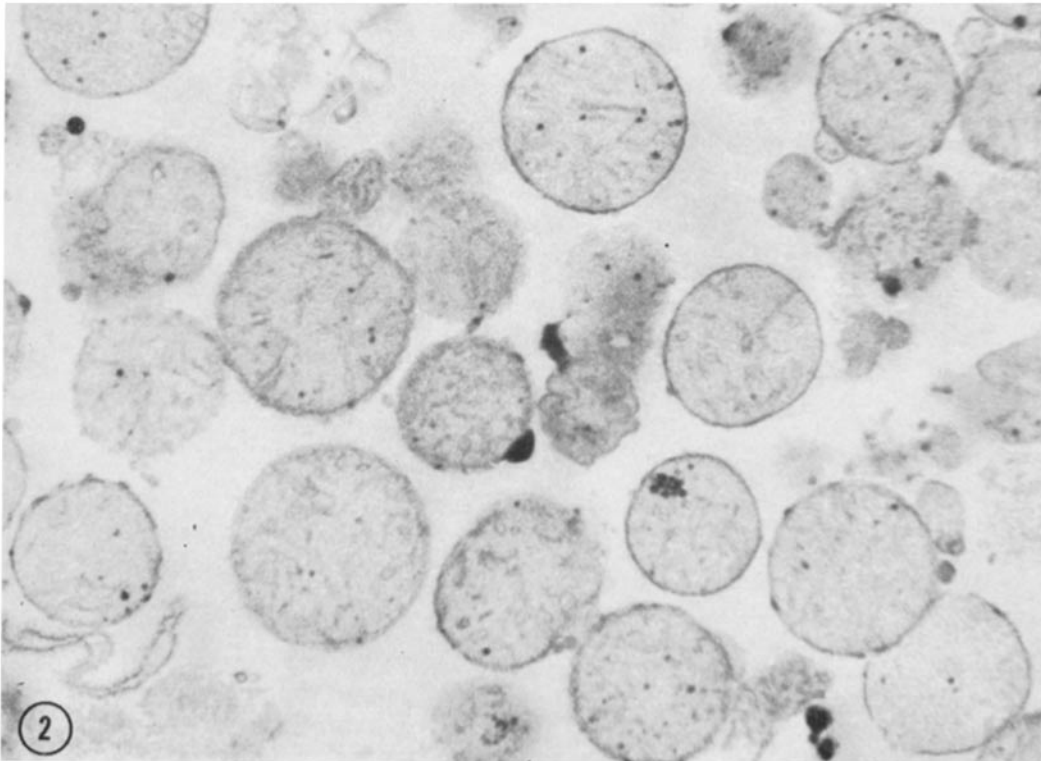
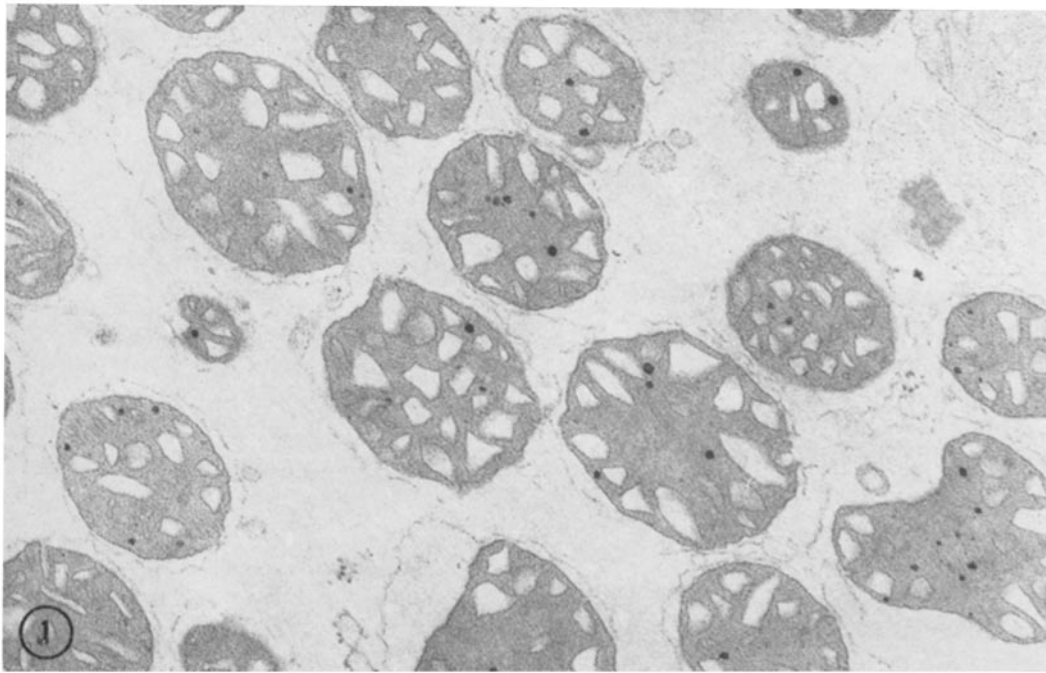


FIGURE 1 Rat liver mitochondria isolated in 0.25 M sucrose and washed three times. Note the spherical outline and the dilated cristae. Stained with lead citrate. $\times 32,000$.

FIGURE 2 Mitochondria isolated in 0.25 M sucrose, fixed in 1% glutaraldehyde for 2 min, washed in sucrose and incubated in GOT medium for 15 min. No enzymatic reaction product is demonstrated. Unstained. $\times 29,000$.

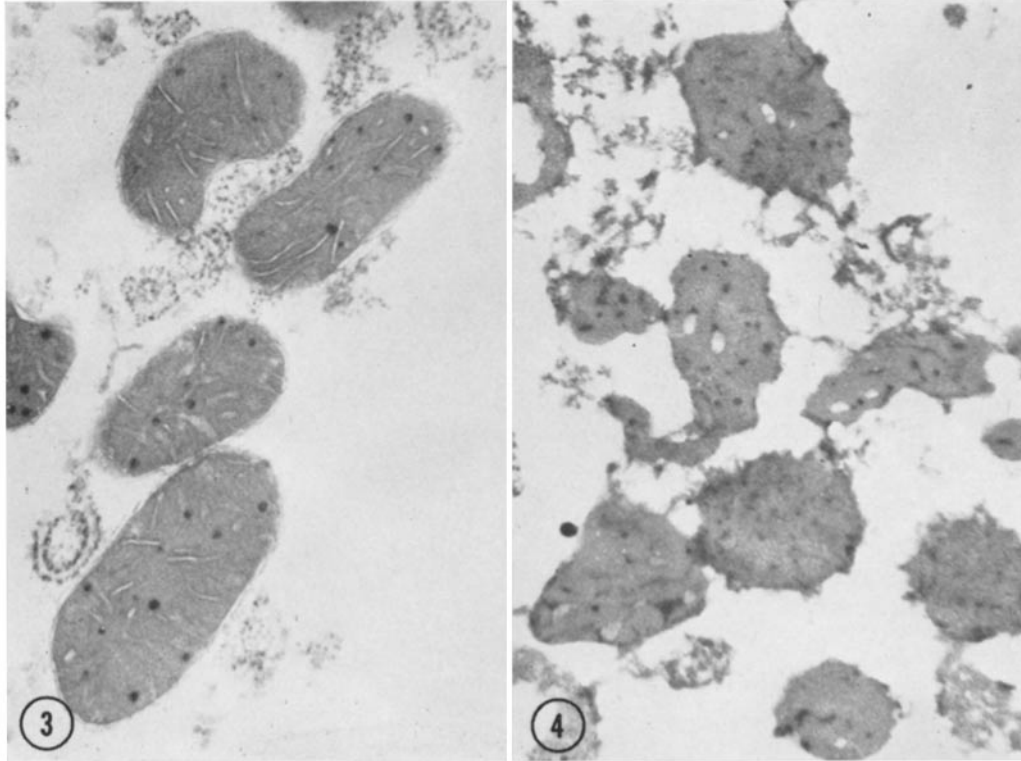


FIGURE 3 Mitochondria isolated in 7.3% PVP-0.25 M sucrose and fixed in 1% glutaraldehyde for 2 min. Their appearance is more compact than those shown in Figs. 1 and 2. No enzymatic reaction product is demonstrated after the pellet is incubated for GOT activity. Stained with lead citrate. $\times 32,000$.

FIGURE 4 Mitochondria isolated in 0.25 M sucrose, redispersed in a solution consisting of 0.125 M KCl, 0.01 M ATP and 0.02 M Tris-HCl, pH 7.4, and fixed in 1% glutaraldehyde for 2 min. Their outline is irregular and the space of the cristae appears to reduce considerably. No enzymatic reaction product is demonstrated in the mitochondria after the pellet is incubated for GOT activity. $\times 25,000$.

RESULTS

Electron Microscope Study of GOT Activity of the Mitochondria Fixed In Situ and In Vitro

The unfixed liver tissue mitochondria isolated in isotonic sucrose assumed a spherical outline (Fig. 1) rather than their usual elongated shape seen in an intact cell. In addition, both the space between the external membranes and the cristae appeared to be dilated. No enzymatic activity was demonstrated histochemically after fixation (Fig. 2).

The addition of PVP in the sucrose solution resulted in mitochondria which had a more compact appearance (Fig. 3). The contraction of the mitochondria was successfully induced by

ATP (Fig. 4). However, no GOT activity in these mitochondrial pellets was demonstrated by the histochemical method.

After the liver tissue was perfused briefly with a glutaraldehyde-formaldehyde mixture, it was possible to isolate the hepatic mitochondria from the fixed tissue by differential centrifugation. The mitochondria in the fraction were not accompanied by an undue amount of contaminants and retained their slender and compact appearance as often seen in an intact cell, although the surface membranes peeled away at places (Fig. 5). After a subsequent *in vitro* fixation in formaldehyde, the mitochondrial GOT activity was demonstrated by histochemical means and the

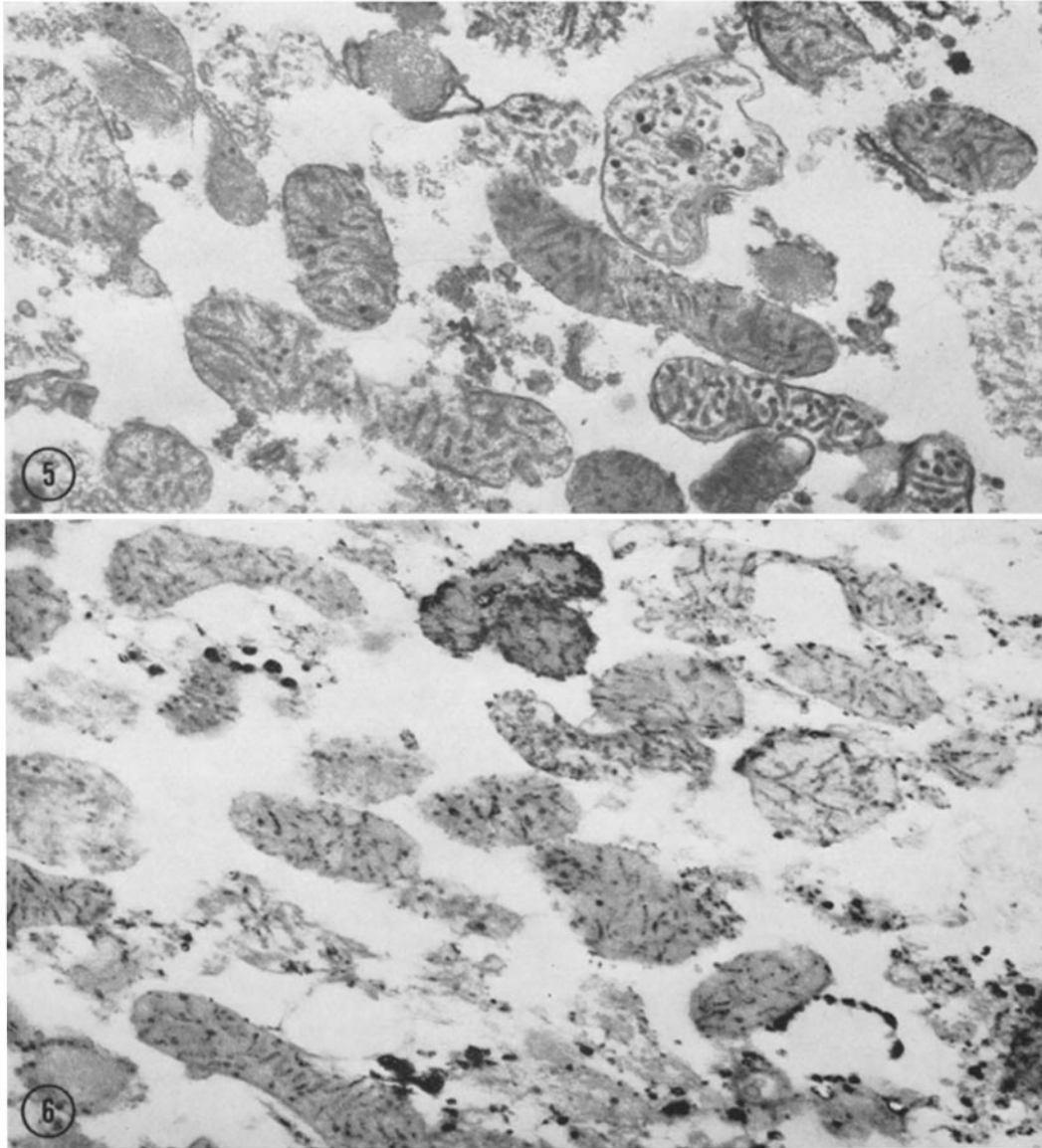


FIGURE 5 Mitochondria of rat liver perfused *in situ* with a glutaraldehyde-formaldehyde mixture. After being isolated in 0.25 M sucrose, they retain their slender and compact appearance although the surface membrane peels away at many places. Stained with lead citrate. $\times 36,000$.

FIGURE 6 Mitochondria of rat liver perfused *in situ* with glutaraldehyde-formaldehyde mixture. After being isolated in 0.25 M sucrose, they were fixed in 3.7% formaldehyde for an additional 30 min, washed, and incubated in GOT medium for 15 min. Note that enzymatic reaction product is largely present in the cristae. Section unstained. $\times 34,000$.

enzymatic reaction product was largely localized to the cristae (Fig. 6), similar to the pattern of distribution in the mitochondria of an intact cell. There appeared to be little reaction product deposited at the mitochondrial surface. The absorption spectrums (Fig. 7) of the hydrazone formed by the reaction product were typical for oxalacetic acid.

Assays of GOT Activity of the Mitochondrial Fractions Fixed In Situ and In Vitro

The GOT activity in the mitochondrial fractions isolated and fixed in different states is presented in Table I. After perfusion with aldehyde fixative, a significant amount of GOT activity was preserved in the mitochondrial suspension. About 62% of the initially preserved activity was able to survive an additional treatment of 3.7% formal-

dehyde for 30 min. An increase in GOT activity was evident in the presence of 6 mm lead nitrate both with unfixed mitochondria and with mitochondria fixed by perfusion followed by immersion method.

The GOT activity of the hepatic mitochondria isolated in isotonic sucrose, in PVP-sucrose, or contracted by the addition of ATP did not survive the fixation procedure of glutaraldehyde or formaldehyde.

DISCUSSION

These assays of GOT activity reveal the paradoxical fact that, while the histochemically demonstrable GOT of fixed mitochondria can be assayed biochemically, the GOT activity of fresh mitochondrial fractions cannot be demonstrated by a histochemical method. The latter method is not successful because the enzyme activity of the isolated fresh mitochondria fails to survive a 2 min fixation by glutaraldehyde, whereas considerable GOT activity remains in the mitochondria of cells which have been perfused *in situ* with a glutaraldehyde mixture. Previous assays of mitochondrial activity after differential centrifugation have demonstrated that the enzyme protein is firmly bound to mitochondrial membranes and that it is

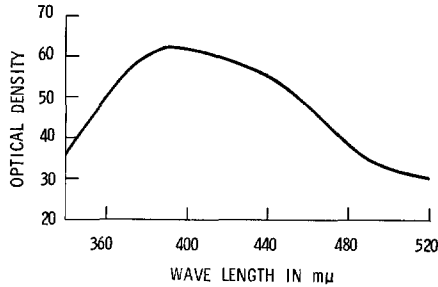


FIGURE 7 The pellet of mitochondria shown in Fig. 6 was incubated in GOT medium for 30 min; the precipitate was treated with 2,4-dinitrophenyl hydrazine in HCl. The absorption spectrums of the hydrazone formed by the reaction product are typical for oxalacetic acid.

TABLE I
Assays of Got Activity (Units/ml) of Mitochondrial Suspension Prepared by Different Means Before and After Fixation

Preparation	In vitro fixation	Karmen method	Modified Reitman-Frankel method	
			Without lead	With 6 mm lead
Perfusion-fixed tissue; isolation in sucrose	None	192	168	340
	Formaldehyde	120	112	234
Unfixed tissue; isolation in sucrose	None	240	216	512
	Glutaraldehyde	0	—	—
	Formaldehyde	0	—	—
Unfixed tissue; isolation in PVP-sucrose	None	168	—	—
	Glutaraldehyde	0	—	—
	Formaldehyde	0	—	—
Unfixed tissue; isolation in sucrose with ATP-contraction	None	204	—	—
	Glutaraldehyde	0	—	—
	Formaldehyde	0	—	—

0 = unmeasurable.
— = not done.

solubilized only with great difficulty (3, 5). Thus the physicochemical orientation of the enzyme protein within the membranes of an intact cell appears to be important for the enzyme to survive the fixation procedure. This concept is enhanced by the finding that an exposure of the purified enzyme to aldehyde fixation destroys its activity (12). Enzyme proteins are generally believed to be located within the bimolecular leaflets of lipids and proteins which comprise a membrane, with only their active groups exposed to one or both surfaces of the membrane (17). The fixation of the membrane lipoproteins adjacent to the enzyme apparently protects the enzyme protein, probably by limiting further access of aldehyde to the enzyme.

Glutaraldehyde (2) as well as formaldehyde (6) has been demonstrated to form cross bridges primarily between amino groups and to stabilize the peptide chains of the membrane components in the tissue. The finding by Levy et al. (15) that glutaraldehyde-perfused brain has relatively normal electrolytes strongly suggests that certain cellular membranes are rendered impermeable. The lack of cell volume change when glutaraldehyde-perfused brain is soaked in anisotonic media also is consistent with this suggestion (19). Formaldehyde does not appear to stabilize the cellular membranes as effectively as glutaraldehyde, and cell swelling is a serious problem in formaldehyde-fixed tissue (1). The finding that tissue GOT briefly fixed in dilute glutaraldehyde is more resistant to the effect of formaldehyde than the GOT of unfixed tissue (11) also implies that glutaraldehyde fixation protects the enzyme from the effects of formaldehyde.

Trauma and storage of tissue in sucrose have been shown to result in less histochemically demonstrable GOT activity in rat liver cells (13). Both conditions also appear to have an effect upon membrane permeability. In electron micrographs of traumatized and stored tissue the altered membrane permeability is chiefly manifest by swelling. In reality this situation is duplicated during the preparation of the mitochondrial fraction by differential centrifugation. Both trauma and sucrose storage are involved and swelling is characteristic of the isolated mitochondria. If this altered membrane can no longer be effectively cross-linked by glutaraldehyde to form an impermeable structure, the enzyme protein would be vulnerable to the further effect of fixation. The swelling per se does not appear

destructive since the addition of PVP or ATP into the isolation or wash fluid results in more compact mitochondria, as pointed out by Novikoff (16) and Lehninger (14), but does not prevent the enzyme from being inactivated by the fixatives.

The ultrastructural appearance of the mitochondria isolated in isotonic sucrose varies. The mitochondria resemble more closely those seen in an intact cell if isolated with a rapid technique (4), but usually they assume a rather spherical condensed conformation (7). In the present study, the condensed conformation is often encountered when the mitochondria are fixed after being washed (Fig. 1), while an orthodox conformation (7) prevails when they are fixed immediately after separation (Fig. 2). There appears to be no significant difference in preservation of GOT activity histochemically between the two conformations.

The measurable GOT activity of the mitochondria isolated from the aldehyde-perfused liver is remarkably high, equivalent to about 80% of that of the unfixed mitochondrial fraction. This seems to indicate that the membrane effect of glutaraldehyde fortunately occurs before an effect on enzyme protein. The presence of proportionally less activity in these fractions is expected since, although electron micrographs reveal slender mitochondria, the number of the mitochondria in this fraction probably is less than in the unfixed fraction. Undoubtedly homogenization of fixed tissue is less complete than that of fresh tissue. Therefore, more mitochondria would be contained in larger cellular particles or in unbroken cells which were discarded in the nuclear fraction. After an incubation to demonstrate GOT activity, the enzymatic reaction product is well localized in the cristae. This distribution pattern of the reaction product is similar to that seen in mitochondria of the intact cells (13), except that in the fraction the enzyme activity at the surface of the mitochondria is markedly reduced. This latter phenomenon is probably due to a lack of protection of the surface enzyme from the effect of formaldehyde after a mechanical damage of the external membrane of the mitochondria has been induced during homogenization of the tissue.

The modified Reitman-Frankel method is used for GOT assays when the study of the lead effect on enzyme activity is carried out, since it depends neither on a second enzyme system which may be inhibited by lead, nor on the presence of DPN

which may be precipitated by lead ion. With 6 mM lead nitrate incorporated in the medium, the OD values of the hydrazones at wave length near 510 $m\mu$ are consistently higher than the controls. This could be due to an activation of the enzyme by lead ion or to a nonenzymatic conversion of oxalacetic acid into pyruvic acid, the hydrazone of which gives rise to a higher OD than that of oxalacetic acid. As the nonenzymatic decarboxylation of oxalacetic acid at or above pH 7.3 is negligible (12), a true activation of GOT by lead ion appears to be more likely. However, this result does not indicate that there is an actual activation of GOT by lead ion during histochemical study of this enzyme. On the contrary, 6 mM lead nitrate exerts some inhibitory effect on

the enzyme while a concentration of 3 mM lead increases the activity when the histochemical GOT medium is employed for enzyme assays (12). Since the concentration of L-aspartic acid used in the modified Reitman-Frankel method is five times that of the histochemical medium, a much larger portion of the lead could be expected to be chelated by aspartate. Therefore, the activating effect of 6 mM lead probably is due to a reduction in the effective concentration of lead ion.

This work was supported by grant No. NB 04161-05 from the National Institute of Neurological Disease and Blindness of the National Institutes of Health.

Received for publication 12 June 1968.

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