ORIGINAL REPORT

SIGNIFICANCE OF FIXATION IN THE CYTOCHEMICAL DEMONSTRATION OF ORNITHINE CARBAMOYLTRANSFERASE ACTIVITY —WITH SPECIAL REFERENCE TO ALDEHYDE PERFUSION—

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INTRODUCTION

The recent development of a Gomori type method for the cytochemical demonstration of ornithine carbamoyltransferase (OCT) activity has established that the specific activity is located only in the mitochondria of hepatocytes in the rat and mouse (Mizutani, 3, 4). In an earlier paper its cytochemical nature and specificity were described, and it was emphasized that tissue fixation was a critical factor (4). OCT activity survived brief fixation in cold acetone or formol-calcium but not in glutaraldehyde. Although more than 10% of the total activity was preserved with acetone fixation and paraffin embedding, most of the cellular fine structures were damaged. "Sufficient but not excessive" fixation with formal-Ca solution was found to be best. Usually small liver slices cut as thinly as possible were fixed for 4-6 hr at 4°C in 10% buffered formalin, but the central portion of the slices was not always fixed "sufficiently".

The present investigation was undertaken to study the applicability of fixative-perfusion which was expected to fix the tissue evenly and to avoid the difficulties in the localization of OCT activity.

MATERIAL AND METHODS

Preparation of tissue : Male Wistar rats weighing 100-250g were used. The abdomen was opened under light ether anesthsia and the liver was perfused with the cold (4°C) fixative solution through the portal vein. The perfusion pressure was about 80 cmH_2O. Immediately after perfusion started, the caval vein was cut open at its

entry into the thoracic cavity in order to secure a free flow of the fixative through the liver as advocated by Ericsson (1). The following solutions were employed as fixatives; 1) 4% formaldehyde solution obtained from depolymerization of paraformaldehyde (8) containing 1% $CaCl_2$ and adjusted to pH 7.2 with 0.05 M cacodylate buffer; 2) 3% glutaraldehyde adjusted to pH 7.2 with 0.05 M cacodylate buffer (10); 3) a mixture of 4% formaldehyde and 3% glutaraldehyde prepared according to the method of Graham and Carnovsky (2) with reduced concentration of glutaraldehyde.

Fixation times were; with formaldehyde, 10, 20, 30, 45 and 60 min, and with glutaraldehyde or a mixture of formaldehyde and glutaraldehyde, 2, 5, 10 and 20 min. Following fixation, 2-3 mm thick liver slices were washed overnight at 4°C in 0.05 M Tris-maleate or Collidine buffer (pH 7.2) containing 15% sucrose. Frozen sections, 10 μ thick, were used for light microscopic studies and 30 μ frozen sections were employed for electron microscopic observations.

Incubation and observation: The incubating medium consisted of: 0.05 M Tris -maleate buffer (pH 7.2), 4 ml; 1% lead nitrate, 1 ml; distilled water, 5 ml; L-ornithine HCl, 5 mg; carbamoyl phosphate Li salt, 3 mg; and sucrose 0.7 g. The lead nitrate solution was added at last in drops with continuous stirring, and the slightly turbid mixture was filtered and used immediately. Thin frozen sections were incubated for 10-15 min at 18°C, washed in distilled water briefly, immersed in dilute yellow ammonium sulfide, washed again in distilled water and mounted in glycerin jelly.

After incubation, thick frozen sections for electron microscopic studies were postfixed in 1% osmium tetroxide in Veronal buffer, pH 7.4, for 1 hr at 4°C, dehydrated in graded ethanol and propylene oxide, and embedded in Epon. Conversion to sulfide was either performed or omitted before postfixation. The former was used to examine the reactive sites at trimming of the embedded tissues. Ultrathin sections were made with a JUM-5A ultramicrotome and examined with a JEM-7A electron microscope. Most grids were stained with uranyl acetate and lead hydroxide.

In order to compare the results with those of routinely fixed tissues, thin fresh slices were fixed for 4 hr in 4% formaldehyde solution as described earlier (4), followed by the same treatment as the perfusion-fixed sections.

Control experiments: 1) L-ornithine was omitted from the incubating medium or it was replaced by D-ornithine. 2) Sections were pretreated for 15 min with p-chloromercuribenzoate (PCMB) (5 mM, pH 7.2, containing 7% sucrose). 3) Sections were placed in an oven at 90°C for 30 min, and were incubated in the standard medium.

RESULTS

Light microscopic findings: With routinely fixed 5 slices, several sections out of about 50 were incompletely fixed and no adequate localization of the OCT activity was obtained. In other sufficiently fixed sections the specific reaction product was

evenly distributed in the hepatocytes suggesting mitochondrial localization, particularly at the periportal areas.

In sections of tissues perfusion-fixed with formaldehyde for 30 min, there was a marked mitochondrial reaction and good preservation of the cellular structure, although the sinusoids and other blood vessels were dilated (Figs. 1-3). The mitochondrial reaction was tended to be more intense at the periportal than at the centrolobular areas (Fig. 1). In the less reactive cells brown reaction products in small ovoid structures were more prominent, distributed mostly in the peribiliary areas suggesting lysosomal reactions (Fig. 3). This lysosomal staining was also observed in the control sections incubated in the medium from which L-ornithine was omitted or in which it was replaced by D-ornithine (Fig. 4). These findings coincided well with those of our earlier studies with routine formol-fixation (4). In general, the overall activity with the perfusion-fixed tissues for 20-30 min was more intense than with the routinely fixed slices for 4 hr.

With the formaldehyde-perfusion time of 60 min, the mitochondrial activity was markedly suppressed but the lysosomal reaction remained; on the other hand, perfusion time of 10 min resulted in poor fixation and cell damage.

In tissues perfused with glutaraldehyde for 2 min, specific OCT activity in the mitochondria was abolished throughout the hepatocytes, although the cellular structures were well preserved. The lysosomal reaction, however, was retained. In addition, moderate brown reaction products were distributed at the nuclear membranes and perinuclear areas diffusely or in fine filamentous structures (Figs. 5 and 6), particulaly at the periportal areas. This reaction tended to be inhibited by longer glutaraldehyde-perfusion (for 10 min). The control experiments with omission of L-ornithine or with D-ornithine replacing the L-isomer in the incubating medium gave identical results (Fig. 7). Pretreatment of the sections with 5 mM PCMB did not alter the pattern of staining; on the other hand, 0.01 M NaF in the incubating medium suppressed it as it did the lysosomal reaction.

Perfusion with a mixture of formaldehyde and glutaraldehyde for 2 min or longer suppressed both the specific mitochondrial activity, as demonstrated with formaldehyde fixation, and the perinuclear reaction, as observed with glutaraldehyde perfusion, and only lysosomal staining occurred.

Electron microscopic findings: In tissues perfused with formaldehyde for 30 min, the final product of OCT activity, lead phosphate, was distributed in the mitochondrial matrix of the hepatocytes. In the intensely reactive cells, the mitochondrial matrix was filled with electron-opaque fine products, and unstained cristae remained in these products (Fig. 8). Sometimes nonspecific staining occurred in the endoplasmic reticulum and nuclear membrane. In the moderately reactive cells, the localization was discrete and spotty in the matrix and the product sometimes aggregated on the surface of parts of the cristae as indicated in the earlier paper (4) (Figs. 9 and 10). No reaction was noted in the mitochondria of Kupffer cells, epithelium of bile ducts or endothelium of blood vessels. A nonspecific precipitate of lead phosphate

was observed in the lysosomes in various degrees of intensity. A perfusion time of less than 20 min with formaldehyde is not recommended, since cellular fine structures were very poorly preserved.

In tissues perfused with glutaraldehyde for 2-5 min, cellular fine structures were preserved better than with formaldehyde-perfusion for 30 min. However, no or minimum OCT activity was demonstrated in the mitochondria, and deposits of lead phosphate were present in the endoplasmic reticulum and nuclear envelopes (Fig. 11). Some small vesicles closed to the Golgi apparatus also contained the activity (Fig. 12). This staining was also observed in the control sections in the L-ornithine omitted medium (Fig. 12), and abolished by 0.01 M NaF present in the incubating medium or by heat treatment.

With the perfusion with a mixture of formaldehyde and glutaraldehyde, only the lysosomal reaction was noted.

DISCUSSION

The usefulness and merits of vascular perfusion of aldehyde fixative as a way of preserving tissue fine structures for electron microscopy have been described in detail by Pease (8). Ericsson demonstrated glucose-6-phosphatase activity, an unstable enzyme, in the liver after brief perfusion of glutaraldehyde through the portal vein (1). Ornithine carbamoyltransferase is also relatively sensitive to fixatives except for acetone, and, in addition, the liver is one of the tissues into which fixatives can hardly penetrate. The lack of uniformity of histochemical results for OCT activity seems to be due mostly to incomplete or excessive fixation with formaldehyde. The results of the present studies suggest that formaldehyde-perfusion through the portal vein can overcome the disadvantages. Adequate perfusion time was 30 min followed by washing overnight in buffer solution.

The specificity of the uniquely localizing mitochondrial reaction for OCT activity has been discussed in earlier studies (4), and the present study confirmed its intramitochondrial localization, i.e. matrix.

Perfusion with either glutaraldehyde or a mixture of formaldehyde and glutaraldehyde was not effective for the demonstration of OCT activity. It was an unexpected finding that moderate staining occurred in the endoplasmic reticulum and nuclear envelope but none or only very slight staining in the mitochondria with brief glutaraldehyde-perfusion. This extramitochondrial reaction was not by OCT activiy since it was also obtained in the absence of L-ornithine in the incubating medium. There are three possible explanations for this reaction; 1) nonenzymatic staining caused by spontaneous decomposition of carbamoyl phosphate; 2) nonspecific enzymatic hydrolysis of carbamoyl phosphate, probably by acid phosphatase in the lysosomes and other organelles; 3) specific enzymatic hydrolysis such as by glucose -6-phosphatase or acetyl phosphatase. The problem of lead-catalyzed hydrolysis of carbamoyl phosphate and adenosine triphosphate has already been discussed (4,

5, 6, 7 and 9). In the present case, the first possibility can be excluded since the staining reaction was suppressed by longer fixation with glutaraldehyde or by brief fixation with a mixture of formaldehyde and glutaraldehyde, by the addition of 0.01 M NaF to the incubating medium, and by heat treatment of the sections. These findings suggest, rather, an enzymatic reaction, and further investigations are underway to explain this reaction.

SUMMARY

Rat liver was perfused with formaldehyde, glutaraldehyde or a mixture of formaldehyde and glutaraldehyde for a various periods of time from 2 to 60 min; the sections were stained for ornithine carbamoyltransferase activity and examined by light and electron microscopy. In the formaldehyde-perfused sections for 30 min the specific enzyme activity was demonstrated in the matrix of the hepatocytes, and these sections gave more reliable results than those of routinely fixed slices. After brief perfusion with glutaraldehyde, the specific mitochondrial reaction was almost abolished, but the endoplasmic reticulum and nuclear envelope were stained. Further studies are underway to investigate the latter staining. The mixture of 2 aldehydes resulted in demonstration of the lysosomal reaction only.

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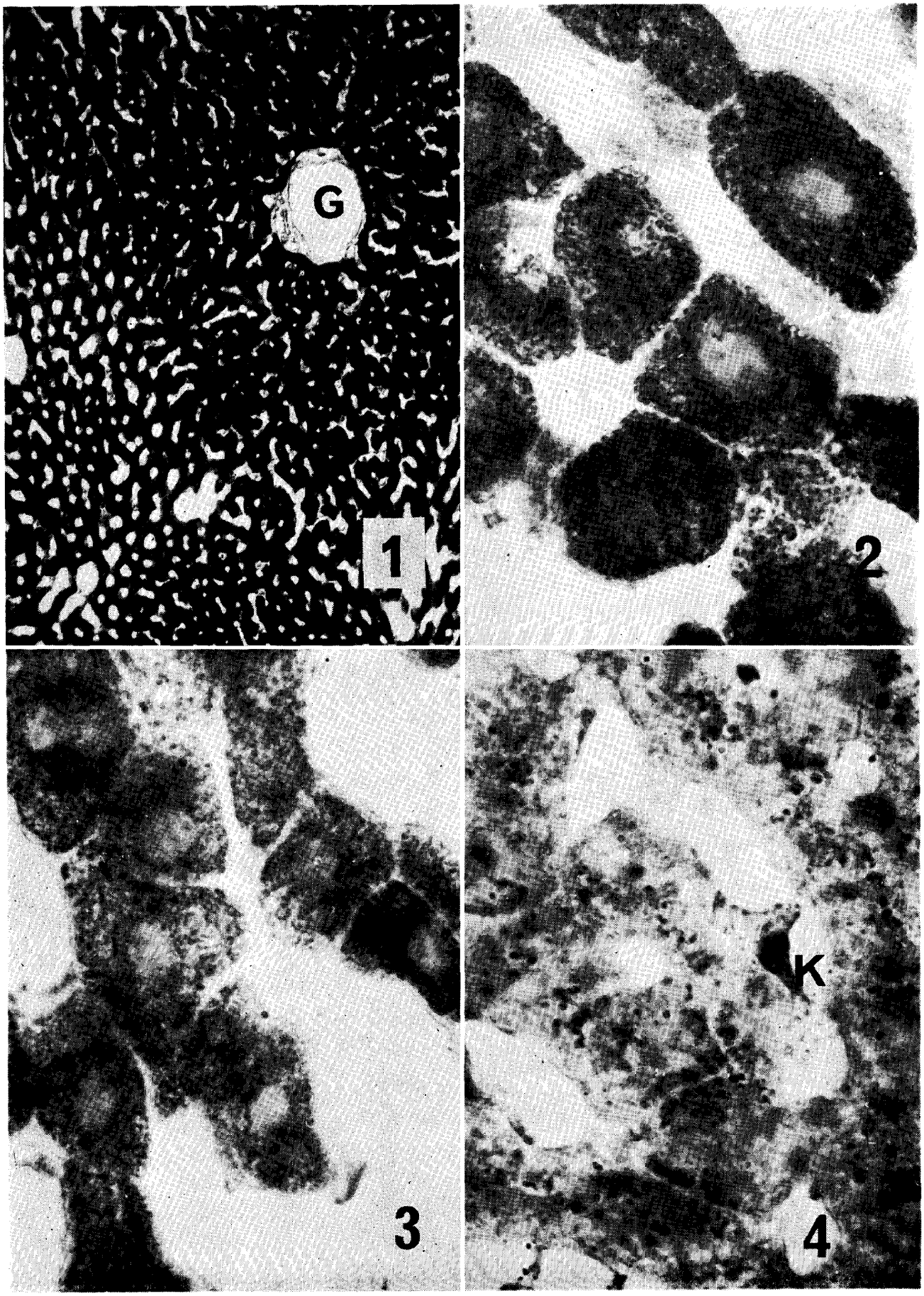
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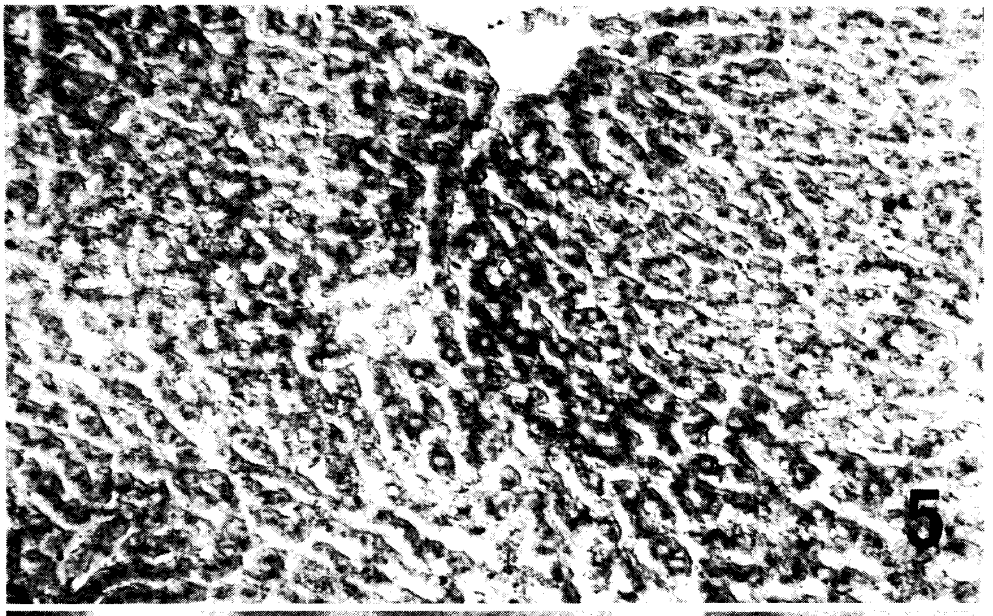
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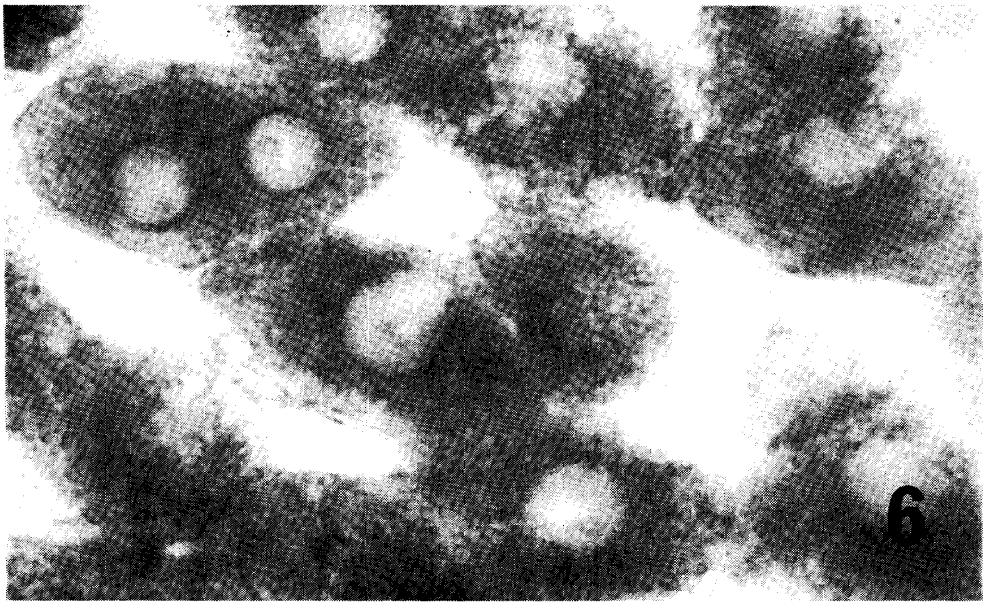
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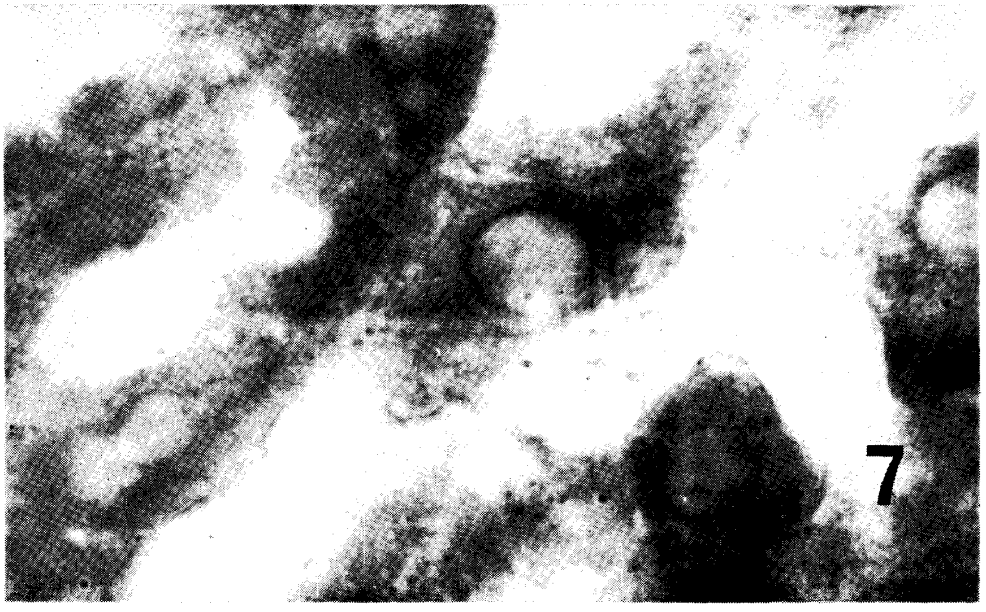
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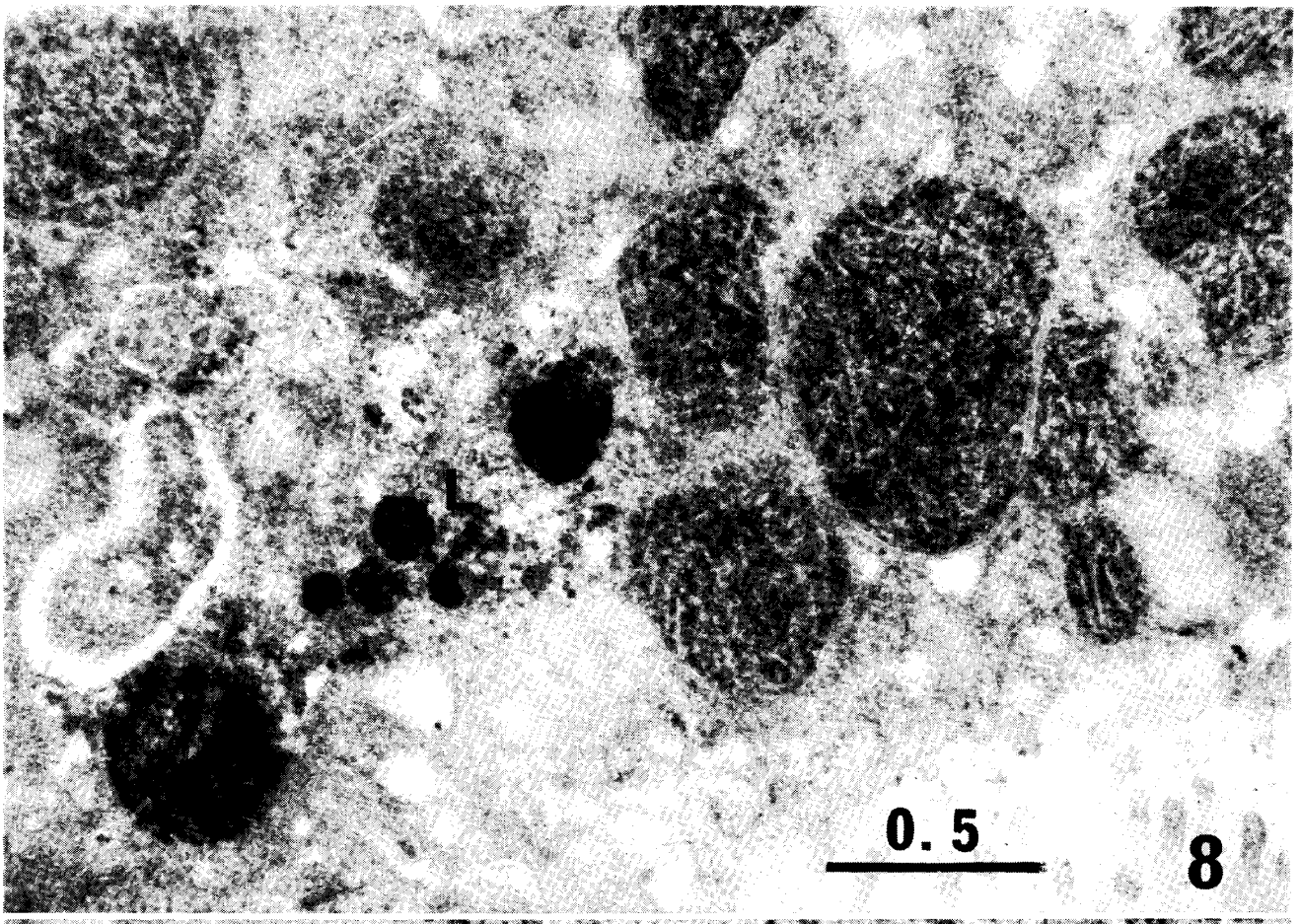
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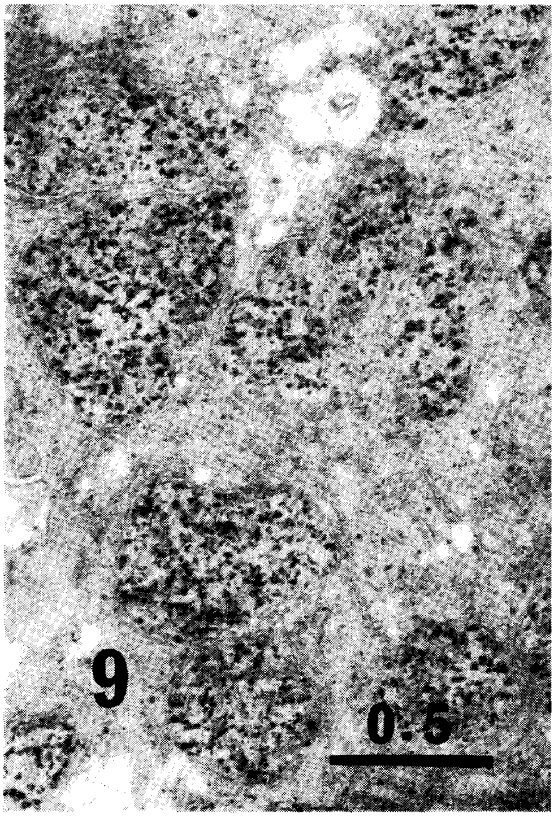
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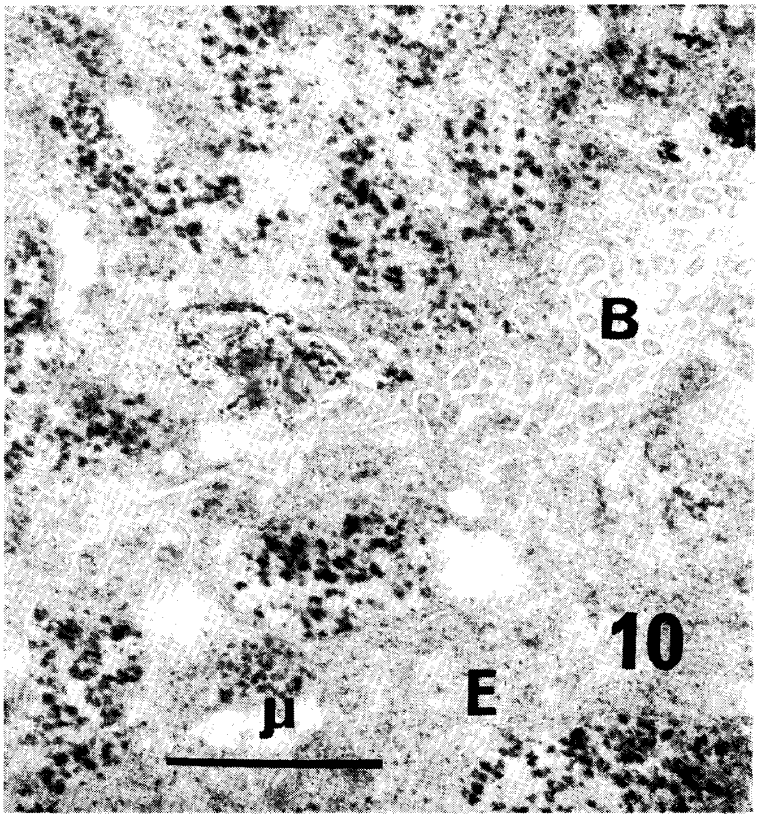
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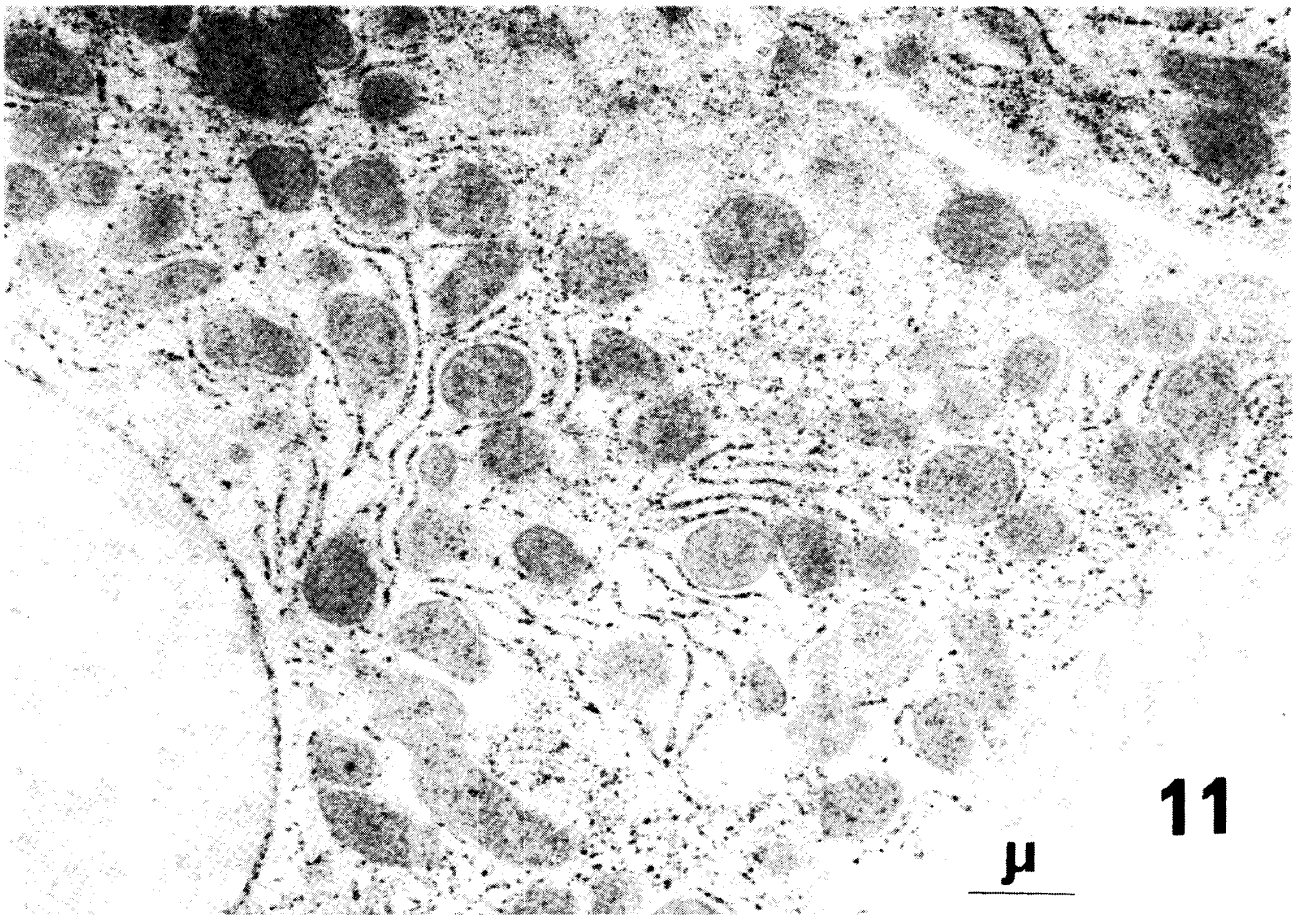
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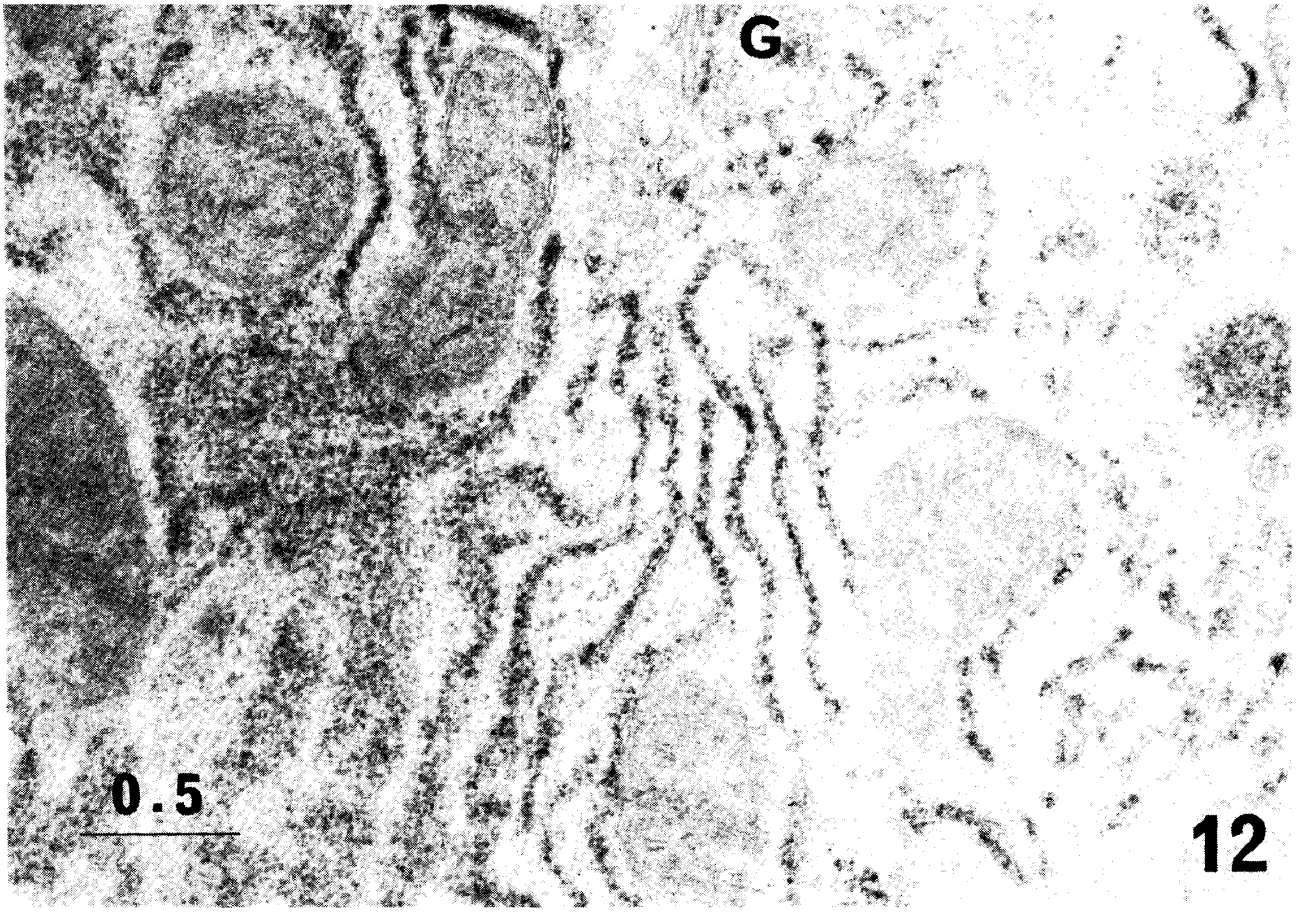
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LEGENDS

Figs. 1–7. Light micrographs.

Fig. 1 Formaldehyde-perfused rat liver for 30 min. The section was incubated for 15 min at 18°C for OCT activity. Marked activity is shown particularly at the periportal area. (G) indicates the Glisson's capsule. ×100

Fig. 2 Higher magnification of Figure 1 is shown. Fine granular and rod shaped reaction product distributes evenly throughout protoplasm of the hepatocytes, suggesting mitochondrial staining. ×1,000

Fig. 3 Relatively less intense reaction are shown. Moderate mitochondrial reaction and more intense lysosomal staining are observed. ×1,000

Fig. 4 A control experiment. The section obtained from formaldehyde-perfused tissue for 30 min was incubated for 15 min in the medium from which L-ornithine was omitted. The reaction is observed in the lysosomes but not in the mitochondrial areas. (K) shows a reaction of the Kupffer cell. ×1,000

Fig. 5 Glutaraldehyde-perfused rat liver for 2 min. The section was incubated for 15 min at 18°C for OCT activity. Moderate lysosomal reaction and slight or moderate perinuclear staining are noted, but no mitochondrial staining is shown. ×100

Fig. 6 Higher magnification of Figure 5 is shown. Diffuse or filamentous staining, particularly at the perinuclear areas, is demonstrated and the nuclear membranes are also stained. ×1.000

Fig. 7 Glutaraldehyde-perfused section was incubated for 15 min in the control medium as Figure 4. The patterns of staining did not alter as compared with Figure 6. ×1,000

Figs. 8–12. Electron micrographs.

Fig. 8 Formaldehyde-perfused tissue for 30 min. The 30 μ frozen section was incubated for 15 min at 18°C for the OCT activity. The mitochondrial matrix of a hepatocyte is filled with the reaction product and unstained cristae are observed. Lysosomes (L) are also markedly stained. ×50,000

Fig. 9 Identical treatment with Figure 8. Less intense mitochondrial reaction is shown. Fine granular deposits distributed evenly in the matrix. ×37,500

Fig.10 Same as Figure 9. No activity is noted in the endoplasmic reticulum (E) and bile canalcules (B). ×25,000

Fig.11 Glutaraldehyde-perfused tissues for 2 min. The 30 μ section was incubated for 15 min in the standard medium for OCT. No or very slight activity is shown in the mitochondria. The reaction occurred in most of the endoplasmic reticulum and nuclear envelope. ×12,500

Fig.12 Glutaradehyde perfused section was incubated for 15 min in the control medium as Figure 4. No reaction product is observed in the mitochondria, but the endoplasmic reticulum and small vesicles closed to the Golgi apparatus (G) contain the products. ×37,500