ELECTRON MICROSCOPE STUDIES ON ENZYME ACTIVITY AND THE ISOLATION OF THIOHYDANTOIN-INDUCED MYELIN FIGURES IN RAT LIVER

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ABSTRACT

Characteristic cytoplasmic inclusions (myelin figures), consisting of concentric multilaminar paired membranes surrounding one or more lipid bodies, were produced in rat liver parenchymal cells by incorporating high doses of an anticonvulsant agent (Bax 422Z) into the animals' diet. Enzymatic reaction product (presumably lead phosphate) was found around the central fat of these myelin figures in liver which had been fixed in glutaraldehyde, incubated in Wachstein and Meisel's medium containing adenosine triphosphate or inosine tri- or diphosphate, postosmicated, embedded in epoxy resin, and examined in the electron microscope. In an attempt to isolate myelin figures, fresh liver from medicated rats was homogenized and differentially centrifuged. Thin sections of osmium tetroxide-fixed, Epon-embedded pellets from each fraction were examined with the electron microscope. The concentric membranous whorls, which are probably derived from cisternae of the endoplasmic reticulum, broke up as the cells were disrupted and became inextricably mixed with the microsomal fraction. However, when liver previously fixed in formalin for 24 hours was homogenized, the myelin figures remained intact.

INTRODUCTION

Characteristic cytoplasmic membranous whorls have been observed recently in the enlarged livers of rats following treatment with high doses of the anticonvulsant agent, 3-allyl-5-isobutyl-2-thiohydantoin (Bax 422Z), and also after medication with phenobarbital (1–3). These cytoplasmic inclusions, which we refer to as myelin figures, have a diameter of up to 8 μ, and consist of a whorl of up to 80 concentric paired membranes surrounding one or more central lipid bodies and a variable amount of vesicular material. The membranous whorl appears to be derived from the rough endoplasmic reticulum (ERR), with a loss of ribosomes from most of the cisternae making up the myelin figure (2).

Similar cytoplasmic inclusions have been observed previously in abnormal liver parenchymal cells, but usually have been associated with necrosis, inflammation, or malignant change (4–14), none of which is induced by Bax 422Z or phenobarbital (2, 3).

Recent speculation on the significance of such myelin figures (13, 14) prompted the present investigation, which was designed to elucidate two points. First, whether enzymatic activity could be demonstrated in these myelin figures; in view of the frequently observed association of nucleoside-phosphatase activity with various membranes of many cells (15), adenosinetriphosphate and inosine tri- and diphosphate were chosen as sub-

strates. Secondly, whether these cytoplasmic inclusions could be separated from other cellular organelles by fractionation and differential centrifugation. The present communication reports on the localization of nucleoside phosphatase reaction product within myelin figures of intact hepatocytes, and on the results of attempts to separate these inclusions from fresh and formalin-fixed liver.

MATERIALS AND METHODS

Pronounced liver enlargement was induced in a group of ten young male Sprague-Dawley rats by incorporating 0.5 per cent w/w Bax 422Z into their diet for 3 months, in the manner previously described (2). The animals were killed by cervical dislocation; the average relative weight of their liver was 6.9 gm per 100 gm body weight, compared with 2.9 gm per 100 gm in a group of 5 unmedicated control rats. Portions of the enlarged livers, and in some cases of the unmedicated control livers, were used for the following procedures.

GENERAL MORPHOLOGY: Thin slices of liver were fixed in aqueous 10 per cent formalin buffered to pH 7.0 with phosphate, and were embedded in paraffin; sections were cut, stained with hematoxylin and eosin, and examined with a light microscope. Small pieces of liver (less than 1 cubic mm) were cut under and fixed in cold phosphate-buffered osmic acid (16) for 1 hour, after which they were processed and examined by light and electron microscopy as described below.

CYTOCHEMICAL PROCEDURES ON INTACT HEPATOCYTES: Preliminary experiments with frozen sections of treated liver revealed severe alterations in the morphology of the myelin figures, and therefore it was decided to use small blocks of tissue which following fixation were more finely diced prior to incubation, after the manner of Marchesi and Barrnett (17). Thus, pieces of liver less than 1 cubic mm in size were fixed for 2½ hours at 4°C in 5 per cent glutaraldehyde buffered to pH 7.4 with 0.2 M sodium cacodylate, after which they were washed overnight at 4°C in cacodylate buffer, pH 7.4, containing 7.5 per cent sucrose (18). These small pieces were then more finely diced, and incubated for 35 minutes at 37°C in the lead-containing medium of Wachstein and Meisel (19) with adenosine triphosphate (ATP), inosine triphosphate (ITP), or inosine diphosphate (IDP) as substrate. Two controls were used: osmium tetroxide–fixed tissue was incubated in the complete media, and glutaraldehyde-fixed material was incubated in media from which only the nucleotide substrate was lacking. Following incubation, the pieces of tissue were washed briefly in buffer, and were then treated with osmium tetroxide for 45 to 60 minutes at 4°C.

Studies on Liver Homogenates

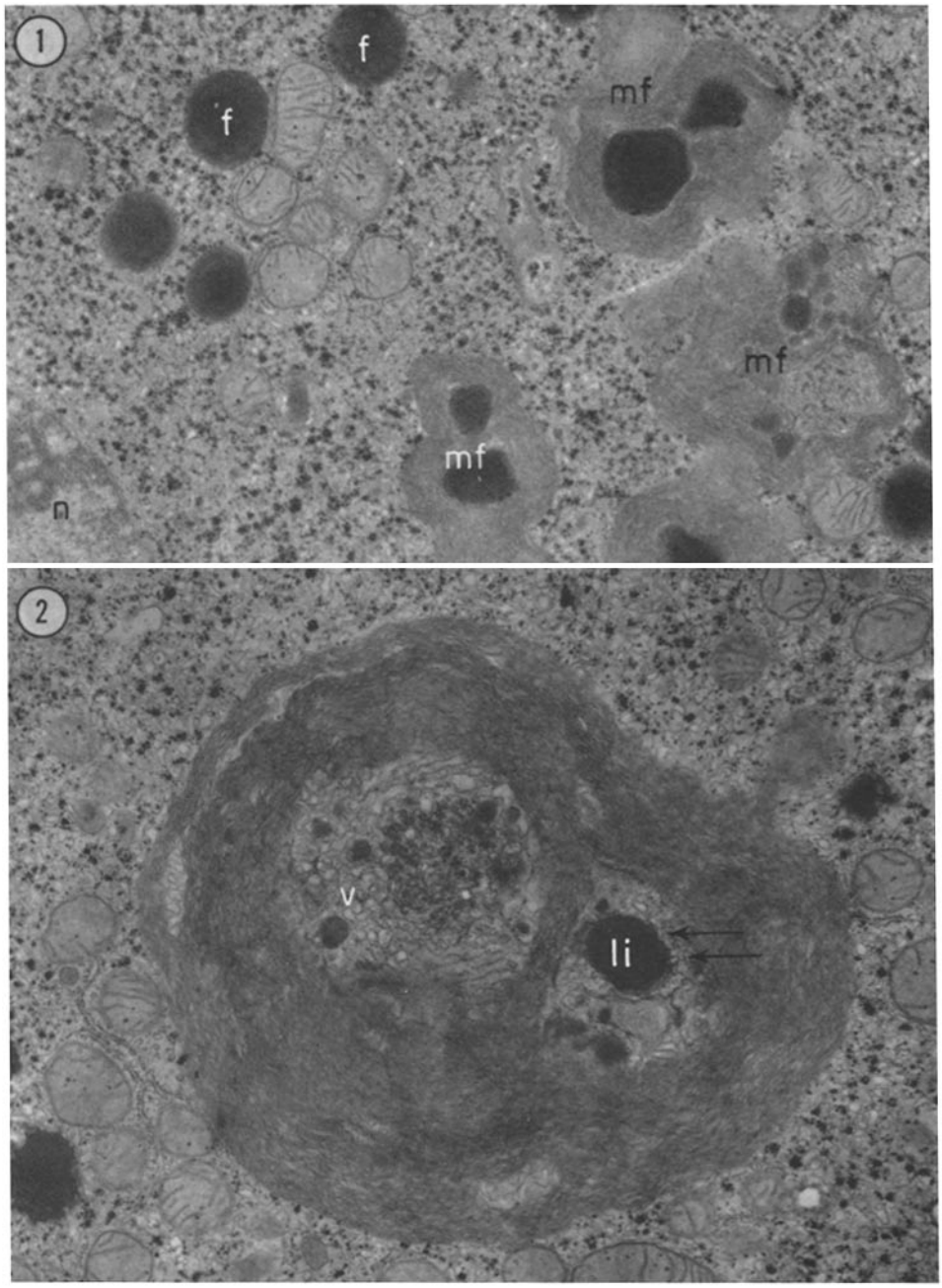
FRESH LIVER: Homogenates of fresh liver in 0.25 M sucrose were prepared using the method of Hogeboom (20), and examined by phase-contrast microscopy. Then nuclear, mitochondrial, and microsomal fractions were obtained by differential centrifugation in 0.25 M and 0.34 M sucrose (20). Pellets of each of these fractions were made by centrifuging the material through cold phosphate-buffered osmic acid (16), essentially in the manner of Epstein (21), but using microcentrifuge tubes as suggested by Malamed (22). The pellets were cut into small cubes under 50 per cent alcohol. Additional pellets of the nuclear fractions were prepared by the same method, but were centrifuged through cold cacodylate-buffered glutaraldehyde, and cut into small cubes under cold sucrose-cacodylate buffer (18). These cubes were stored overnight at 4°C in the same buffer. They were then cut into even smaller pieces, and incubated in the Wachstein and Meisel media at 37°C for 35 minutes, using ATP, ITP, or IDP as substrate, after which they were then treated with osmium tetroxide for 30 to 45 minutes. Controls were prepared as described for whole tissue.

FORMALIN-FIXED LIVER: Homogenates in 0.25 M sucrose were made of liver slices previously fixed for 24 hours in 10 per cent formalin buffered with phosphate to pH 7.0. The homogenates were examined by phase-contrast microscopy, and were then centrifuged at 700 *g* for 10 minutes. Three layers were obtained, and pellets from each of these fractions were prepared by spinning through osmium tetroxide as described above. Small cubes were cut from the pellets under 50 per cent alcohol.

PREPARATION OF SPECIMENS FOR LIGHT AND ELECTRON MICROSCOPY: All the small blocks from these various procedures were dehydrated through graded alcohols and embedded in Epon 812 (23). Sections were cut with a Porter-Blum Mark I ultramicrotome using glass knives. For light microscopy, sections 0.5 μ thick from each block were mounted on glass microscope slides and stained with toluidine blue (24). In addition, similar sections from incubated material were treated with ammonium polysulphide in order to assess the distribution of lead reaction product throughout the tissue. For electron microscopy, ultrathin sections were mounted on plain Effa or Athene copper grids; incubated material was, for the most part, left unstained, whilst other tissue was stained with lead acetate (25). The sections were examined in an RCA EMU 3c electron microscope.

OBSERVATIONS

GENERAL MORPHOLOGY: Livers from rats which had received Bax 422Z for 3 months showed, by light and electron microscopy, morphological



All the figures are electron micrographs of Bax 422Z-treated rat liver. Figs. 1 to 3 are of material fixed in osmium tetroxide in the usual manner.

FIGURE 1 Part of an hepatocyte in which portions of several myelin figures (*mf*) are present. There is a marked lack of orientated profiles of cisternae of the rough endoplasmic reticulum, and an abnormal increase in the amount of smooth endoplasmic reticulum. Glycogen particles are prominent, and there are fat droplets (*f*) in the cytoplasm unassociated with the membranous whorls of myelin figures. Nucleus (*n*). × 10,000.

FIGURE 2 Part of an hepatocyte showing the appearance of a myelin figure. The multilaminar whorl is made up of concentric paired smooth membranes. Centrally, there is a considerable amount of vesicular material (*v*) and some apparently free ribosomes (arrows). Lipid body (*li*). The smooth endoplasmic reticulum is prominent in the cytoplasm surrounding the myelin figure. × 14,000.

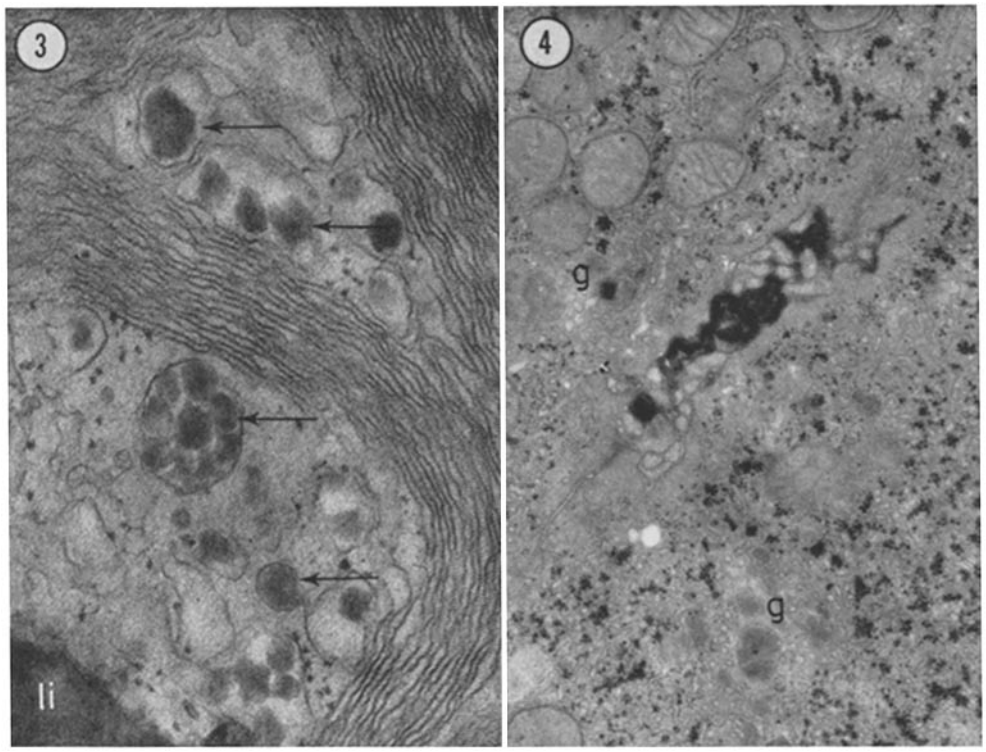
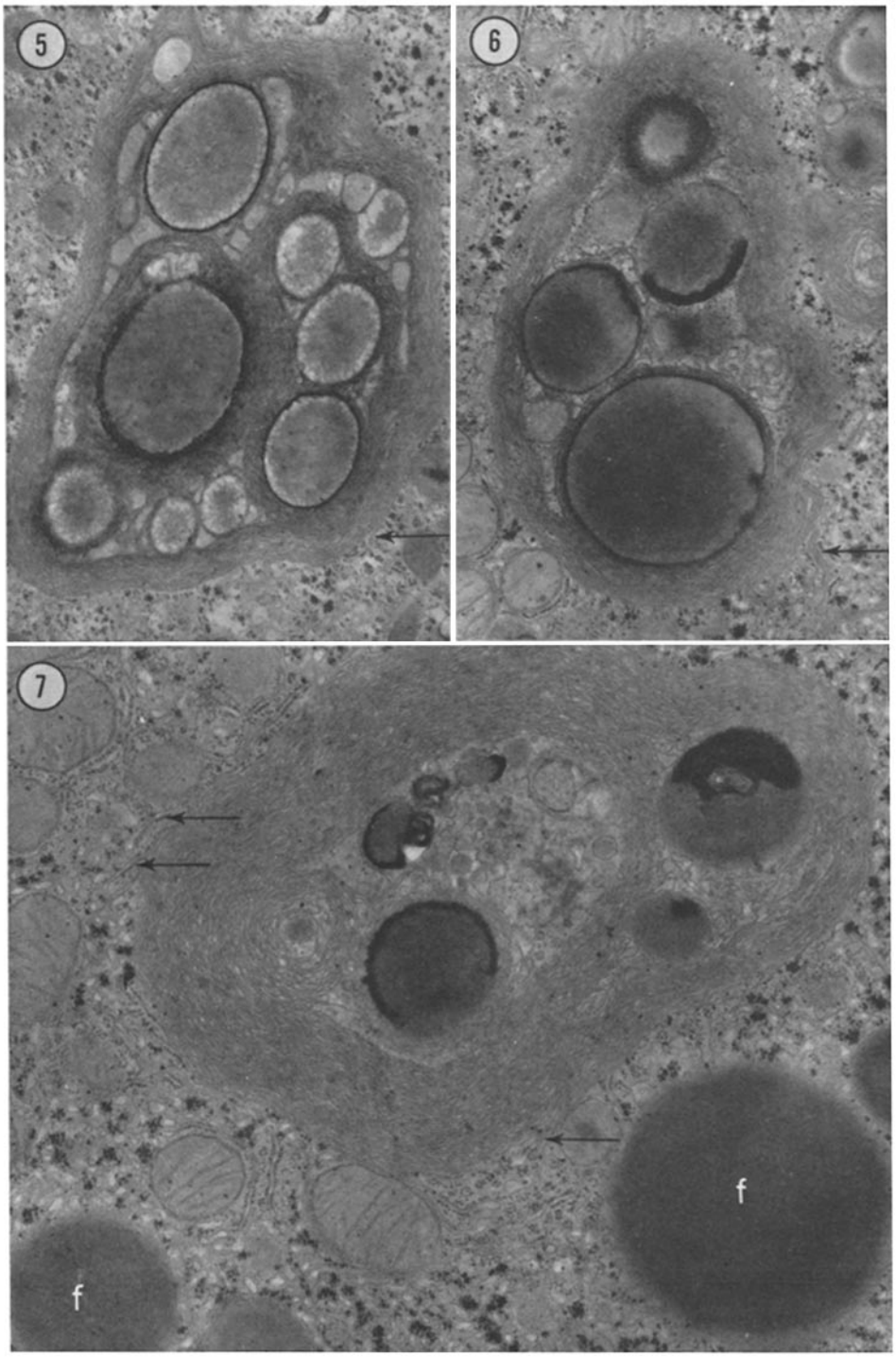


FIGURE 3 Part of a myelin figure in which there are smooth vesicles, containing electron-opaque material (arrows), which resemble those of the Golgi apparatus (Fig.4). The paired smooth membranes making up the whorl are clearly shown, and portion of a central lipid body (*li*) is present. × 56,000.

FIGURE 4 Bile canaliculus and pericanalicular region of two neighboring hepatocytes. This glutaraldehyde-fixed tissue had been incubated for ATPase activity and then treated with osmium tetroxide. Dense reaction product is present in the canaliculus, and the Golgi zones (*g*) are prominent, with many vesicles containing secretory granules. × 18,000.

changes similar to those noted after 1 month of medication (1, 2). Thus, the parenchymal cells were abnormally large, and "light" and "dark" hepatocytes were observed frequently. The rough endoplasmic reticulum (ERR) was dispersed, with swelling and vesiculation of the cisternae, and there was a marked increase in amount of smooth vesicular elements of the endoplasmic reticulum (Figs. 1 and 2). Myelin figures were even more prominent than after 1 month of medication (2). About 50 per cent of the parenchymal cells contained from one to twelve of these cytoplasmic

FIGURES 5 to 7 Myelin figures in glutaraldehyde-fixed liver incubated for nucleoside phosphatase activity and then treated with osmium tetroxide. Fig. 5, ATP as substrate; Fig. 6, ITP as substrate; Fig. 7, IDP as substrate. With each substrate, dense reaction product is present around part or all of the periphery of many of the central lipid bodies. No deposit is seen in fat unassociated with the membranous whorls of myelin figures (*f*, Fig. 7). The arrows denote connections between the peripheral layers of the membranous whorls and the surrounding endoplasmic reticulum. Fig. 5, × 13,000; Fig. 6, × 14,000; Fig. 7, × 21,000.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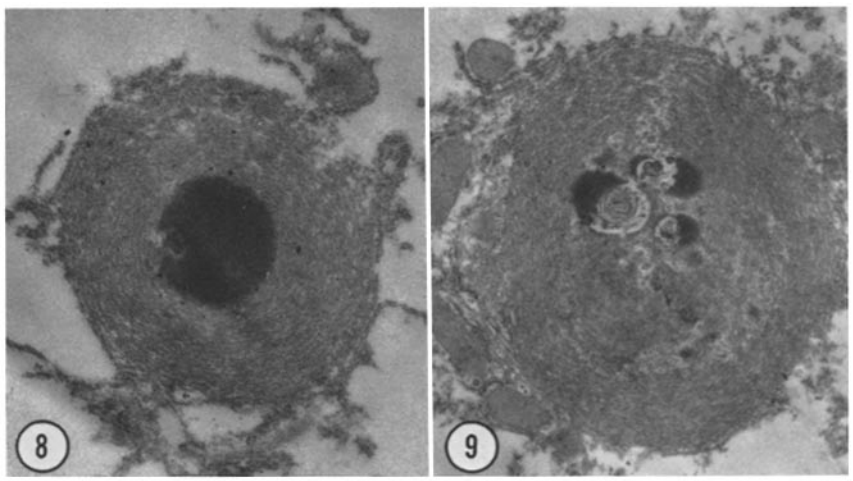

FIGURES 8 and 9 Examples of myelin figures separated from formalin-fixed hepatocytes by homogenization and centrifugation. These myelin figures, which were concentrated in the heaviest layer, are fairly well preserved apart from the central lipid, which in some instances appears shrunken. Fragments of the endoplasmic reticulum and occasional mitochondria are attached to the periphery of some of the membranous whorls. Fig. 8, × 14,000; Fig. 9, × 12,000.

inclusions, some of which had a strikingly complex multiple-whorled structure. Within the "core" of each whorl of paired smooth membranes, there were one or more lipid bodies together with a variable number of smooth vesicles. These vesicles frequently contained discrete dense material similar in appearance to the secretory granules of the Golgi apparatus (Fig. 3).

DICED INCUBATED LIVER: Good preservation of hepatic fine structure, including both membranes and fat of the myelin figures, was achieved with glutaraldehyde fixation followed by enzyme staining procedures and treatment with osmium tetroxide (Figs. 4 to 7).

The myelin figures showed dense enzyme reaction product, presumably lead phosphate, deposited around the periphery of most of their central lipid bodies. The site and type of deposition was the same whether ATP, ITP, or IDP had been used as the substrate (Figs. 5 to 7). By light microscopy, the reaction product was seen to be evenly distributed across most of the sections, though in some instances deposition was more pronounced at the periphery of the block (26). No deposits were seen in either the osmium tetroxide–fixed or substrate-lacking controls, nor did they occur in fat inclusions unassociated with myelin figures (Fig. 7). With all three substrates, bile canaliculi (Fig. 4) and some pericanalicular dense bodies contained reaction product. Sinusoidal surfaces of the parenchymal cells were variably stained after incubation with ATP or ITP but not with IDP, and no deposits were observed in Golgi vesicles, mitochondria, the endoplasmic reticulum, or the membranous whorls of the myelin figures.

HOMOGENIZED FRESH LIVER: With phase-contrast microscopy, the initial homogenates were seen to contain a large number of partially disrupted hepatocytes, together with free nuclei, fat globules, and the usual very small subcellular particles. Nothing resembling a free, intact myelin figure was seen. After differential centrifugation, electron microscopy of the nuclear fraction showed as expected (20) that, in addition to isolated nuclei, there were many partially disrupted hepatocytes. In some of these cells altered myelin figures were noted, in which the concentric paired membranes were much more loosely packed than in intact hepatocytes. No enzyme reaction product was seen in these altered myelin figures, irrespective of whether ATP, ITP, or IDP was used as substrate. No examples of myelin figures isolated from a cell were observed; the mitochondrial and microsomal fractions were of the usual appearance and did not contain any structures recognizably derived from these characteristic cytoplasmic inclusions.

HOMOGENIZED, FORMALIN-FIXED LIVERS: By phase-contrast microscopy the initial homogenates were seen to include variably disrupted hepatocytes and small groups of hepatocytes, together with subcellular debris amongst which were many recognizable "myelin figures". Following centrifugation, electron microscopy revealed relatively well isolated myelin figures in each of the three layers, particularly concentrated in the lower, most dense layer (Figs. 8 and 9). Some of these myelin figures had fragments of ERR and occasional mitochondria attached to the periphery of their multilaminar whorls, but for the most part they were free of other cytoplasmic components. Their morphology was similar to that seen after standard osmium tetroxide fixation of fresh tissue, although in some instances the central lipid was not so well preserved.

DISCUSSION

Enzyme reaction product was found to be localized around the central fat of myelin figures in glutaraldehyde-fixed liver, irrespective of which of the three nucleotide substrates was used. The lack of deposit in both the osmium tetroxide–fixed and nucleotide-lacking control materials discounts this being a mere physical accumulation of lead, although it should be noted that preliminary evidence has been presented to show that lead phosphate deposits may sometimes be formed by the catalytic action of lead on nucleotides (27). Reaction product was not seen around fat unassociated with myelin figures, in contradistinction to the findings of Ashworth, Luibel, and Stewart (28), who noted a dense line of deposit around lipid droplets in formalin-fixed liver treated for ATPase. Furthermore, activity did not persist in the somewhat disorganized myelin figures present after homogenization in partially disrupted cells, even though in such examples the structural relationship between central lipid and the surrounding membranous whorl frequently was preserved.

The present results show, therefore, that relatively non-specific nucleoside di- and triphosphatases are associated with intact myelin figures. However, they do not elucidate the specific nature nor the precise site of such enzyme activity within these cytoplasmic inclusions. Although the reaction product was invariably localized to the periphery of the central fat, this phenomenon could reflect a preferential accumulation of lead phosphate at this location, rather than the actual site of reaction.

In the present study, enzyme reaction product was not observed in the mitochondria, the endoplasmic reticulum or the Golgi apparatus, although other investigators have shown, usually in formalin-fixed tissue, ATPase activity in mitochondria (28–30); ATPase (31, 32) and IDPase (15) activity in the endoplasmic reticulum; and IDPase activity in the Golgi sacs (18). Epstein and Holt (33), in discussing ATPase inhibition in the mitochondria and the endoplasmic reticulum of glutaraldehyde-fixed HeLa cells, have drawn attention recently to the particular care necessary for the demonstration of such enzyme activity in these sites. Under the present experimental conditions, it appears that glutaraldehyde fixation has a similar inhibitory effect on nucleosidephosphatases associated with these organelles, and the lack of reaction product on or between the concentric membranes of the myelin figures could also be due to such a process.

The present study has shown that although the general morphology of myelin figures is preserved within considerably disrupted cells, they cannot be isolated from unfixed hepatocytes by fractionation and differential centrifugation. It appears that after a certain amount of cellular disruption the membranous whorls lose their integrity and behave like the endoplasmic reticulum from which they are probably derived (2), and it may be inferred that their broken-up membranes gravitate with the microsomal fraction. The finding that intact myelin figures can be separated from hepatocytes in homogenates of liver previously fixed in 10 per cent formalin for 24 hours raises the possibility that shorter periods in aldehyde fixatives may preserve the morphology and, in addition, some enzymatic activity of isolated myelin figures. Further work along these lines may help to elucidate whether these cytoplasmic inclusions have a positive function within the cell, or whether they are the end product of some degenerative process.

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