Electron Microscopy on Activity and Localization of Glucose-6-phosphatase in Liver Cells

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It is interesting and in important to study histochemical changes of glucose-6-phosphatase (G-6-Pase) activity by electron microscopy in order to promote the knowledge needed for diagnosis and prognosis in such liver diseases as von Gierke's disease, hepatoma and various other hepatocellular alterations of different origins. Since we had not accomplished the electron microscopic demonstration of G-6-Pase, although light microscopic studies on changes of the enzyme activity were done in this laboratory, this investigation was planned to obtain a satisfactory technique for ultrastructural demonstration of the enzyme activity.

Unfixed frozen sections (80 μ thick) of mouse liver were washed for 2~3 minutes in a 0.4 M sucrose solution (pH 6.8) containing 4 mM lead nitrate and then incubated for 15~20 minutes at 32~37°C in several different media to which 0.4 M sucrose solution was added: A) a modification of the original Chiquoine medium, B) the first modification of the Wachstein-Meisel medium C (the second modification; the 2% lead nitrate solution was reduced in amount to 1.5 ml instead of 3.0 ml in the medium-B). After incubation, these sections were fixed in 1% osmic acid containing sucrose, followed by embedding in Epon, ultrathin-section, mounting and staining with uranyl acetate and/or lead nitrate. By incubating the sections in the medium (B or C), satisfactory preparations were obtainable for its electron microscopic demonstration. The granular deposits of reaction products were found characteristic on the membranous component of the rough-and smooth-surfaced endoplasmic reticulum and unclear envelope.

Occasional deposits were observed within cisternae or vesicles, in the nucleus, and immediately adjacent to the cisternal membrane and glycogen areas.

A specific phosphatase, glucose-6-phosphatase (G-6-Pase), which is amicrosomal enzyme characteristically found in the endoplasmic reticulum, catalyzes the conversion of glucose-6-phosphate (G6P) to glucose. The enzyme removes phosphate from G6P and then free glucose is enabled to diffuse from the cell into the extracellular spaces, including the blood stream. This step is reflected by a rise in the blood sugar. It occurs in greatest amount in liver cells and is also present in the kidney and intestine where it allows these particular
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tissues to add glucose to the blood. However, it is absent from muscles, brain and adipose tissue, and little or none is present in normal serum (Barka & Anderson 1963, Pearse 1968, Harper 1969, Stryer 1975).

In von Gierke's disease, a "glycogen storage disease," the activity of G-6-Pase is either extremely low or entirely absent in the liver. This inherited deficiency of the enzyme causes hypoglycemia, but the liver glycogen is normal in structure and present in abnormally large amounts (Stryer 1975). The activity of liver G-6-Pase may change in the induced hepatocellular alterations (Burstone 1962). Weber & Cantero (1955) found that homogenates of induced rat hepatoma exhibited no G-6-Pase activity, and Latner (1975) described lower levels of the enzyme in patients with cirrhosis. In studies on necrosis of mouse liver in vitro (Griffin et al. 1955), the enzyme activity in liver homogenates showed a significant loss during the early changes leading to cell death. Loss of enzyme activity was demonstrated histochemically (light microscopically) in hepatocytes by administration of hepatotoxins, such as carbon tetrachloride (Chopra et al. 1972) and thioacetamide (Shin et al. 1976). Therefore, it is of interest and there is great need to study histochemical changes of the enzyme activity, especially by electron microscopy, in order to gain knowledge for diagnosis and prognosis in such disorders.

Although light microscopic studies on changes of the enzyme activity were done in this laboratory (Shin et al. 1976, 1977), we have not accomplished an electron microscopic demonstration of G-6-Pase activity. In this connection, it is notable that the staining method is very subtle in details of procedures. Its success depends on various factors, such as proper fixation incubating media and timing, and there is at present considerable difference of opinion as to the method of tissue preparation.

This investigation is planned to obtain a satisfactory technique for ultrastructural demonstration of G-6-Pase activity in liver cells by comparing and/or modifying the referable methods.

**MATERIALS AND METHODS**

Young adult male mice weighing about 20 gm, bred from the same colony in this laboratory, were used and had free access to the laboratory diet and water prior to this experiment.

The unfixed, fresh, frozen sections of the liver (10 μ thick for light microscopy and 80 μ thick for electron microscopy) were washed for 2~3 minutes in a 0.4 M sucrose solution containing 4 mM lead nitrate, titrated to pH 6.8 with NaOH. These sections were incubated in several different media for 15~20 minutes at 32~37°C. For contrast staining preparations, however, small pieces of the liver were prefixed with formalin.

The incubating media were prepared as follows (Tice & Barner 1962, Barka & Anderson 1963, Pearse 1968).

A) a modification of the original (Chiquoine medium) (Chiquoine 1953, 1955).

1. Mixing was done 10 ml of 0.4 M sucrose solution, 4 ml of 0.02 M glucose-6-phosphate solution, distilled water to make 18.8 ml and 1.2 ml of 0.1 M lead nitrate.

2. This mixture was then brought to pH 6.8 with 0.1 N NaOH.

3. After filtration, 6.63 mg of dry lead nitrate were added to the filtrate and the pH was readjusted to 6.85.

B) First modification of the Wachstein-
Meisel medium (Wachstein & Meisel 1956, 1957).

1. A mix was made of 20 ml of 0.125% potassium glucose-6-phosphate solution, 20 ml of 0.2 M tris-maleate buffer (pH 6.7), 3 ml of 2% lead nitrate solution and 7 ml of distilled water.

2. To this mixture was added 6.8 gm of sucrose.

3. The mixture was filtered.

C) Second modification of the Wachstein-Meisel medium. The 2% lead nitrate solution was reduced in amount to 1.5 ml instead of 3.0 ml in the medium B.

D) Medium lacking specific substrate (G6P) for control preparations.

Glucose-6-phosphate in the media (A & B) was omitted.

E) Medium for a second control staining.

The specific substrate (G6P) was replaced with a non-specific substrate, β-glycerophosphate.

For light microscopic demonstration of enzyme activity, after incubation the sections were fixed briefly in 10% formalin after incubation, washed in distilled water and treated with 0.5~1.0% ammonium sulfide solution for 5 minutes. They were treated by rinsing, dehydration and mounting in Histoclad.

For electron microscopy of the enzyme activity, thick sections (80 mμ thick) were transferred directly from the incubating medium to a buffered solution of 1% osmic acid containing sucrose and fixed for 20~30 minutes. After dehydrating in a graded series of alcohol, they were embedded in Epon, cut with glass knives on an ultratome, and stained with uranyl acetate and/or lead citrate.

The degree of enzyme activity and its distribution were estimated by ordinary microscopy and by a Hitachi HE-ll electron microscopy, comparing all the stained sections which had been incubated in different media.

RESULTS

A. Light Microscopy:

In preparations incubated with modifications of Chiquoine (A) and Wachstein-Meisel media (B,C), staining reaction of glucose-6-phosphatase activity was demonstrated and identified properly. The granular deposits of reaction products in the former preparation (A) were intense in staining density and crowded in the cytoplasm (especially around the nucleus), whereas those of preparations incubated with the media (B,C) were slightly weaker in staining.

In these stained sections, brownish-black granular deposits of lead sulfide indicating the sites of enzyme activity were found only in parenchymal cells without nuclear staining. Neither blood vessels nor other cell types occurring in the sections were stained. The distribution of granular deposits in the hepatic lobule was most marked with strongly-staining reaction in liver cells around perportal fields. In the centrilobular zone near the central vein and the intermediate zone of a lobule, enzyme activity was weaker than in the perportal zone, and the granules were relatively dispersed in the cytoplasm (Figs. 1, 2, 3)

Little or no activity was demonstrated when sections were incubated in a medium (D) lacking the substrate (glucose-6-phosphate, G6P) or the medium (E) containing glycerophosphate instead of glucose-6-phosphate, or when sections were incubated after prefixation with formalin. This would indicate that most of the dark granular deposits in the preparations might represent G-6-Phos activity if G6P containing media and unfixed frozen sections
were used.

B. Electron Microscopy:

In ultra-thin sections from the unfixed frozen sections, which were incubated in a modification of Chiquoine medium (A), the granular deposits of final products were coarser and were apt to aggregate (Fig.4), while the granules in sections incubated with modifications (B,C) of Wachstein-Meisel medium were finer and not aggregated (Figs. 5~9). No reaction was seen in the hepatocytes of control preparations incubated in the media (D and E).

The reaction product was found characteristically on the endoplasmic reticulum and nuclear envelope. However, the enzyme activity could not be observed on the plasma membrane, within or on the surface of mitochondria (somewhat disfigured), Golgi complex, dense bodies and other organelles. Furthermore, no reaction product was observable in the general cytoplasmic matrix, except near some of glycogen areas where a few of the granular deposits appeared to be scattered (Fig. 8).

The granules were observed on the membranous component of the rough-surfaced endoplasmic reticulum: the inner and outer surfaces of the cisternal membranes, and outer surfaces of the cisternal membranes, and the membranes themselves. Some of deposits related to the outer surface of cisternae were about the size of ribosomes, but they could be differentiated from ribosomes, in the great majority of instances, because of their stronger electrondensity. The cisternae were considerably dilated and largely empty of contents, but occasional deposits of final product could be observed within cisternae. Sometimes, deposits were encountered immediately adjacent to the cisternal membrane.

The enzyme activity was represented on the surface of small vesicles which seemed to be related to the smooth-surfaced endoplasmic reticulum, and deposits of reaction product were found inside the vesicles occasionally (Figs. 5~8).

The reaction product occurred either on the outer and inner membranes of nuclear envelope or in the perinuclear space between membranes. Occasionally, a few of deposits were also scattered at random among the chromatin (Fig. 5,7).

DISCUSSION

In this investigation on electron-histochemical tests of G-6-Pase activity, the enzyme activity was demonstrated satisfactorily in sections incubated with modifications (B,C) of the original Wachstein-Meisel medium, its localization and specificity being distinct and satisfactory. According to remarks of Novikoff et al. (1958) it was suggested that in the case of adenosine triphosphatase, its activity might be diminished (about 80%) due to the presence of lead ion, so we reduced the concentration of lead nitrate in the medium (C). It is notable that this reduction of the concentration did not hamper good results.

Fine and electron-denser granules of the reaction product were well defined and were deposited on the endoplasmic reticulum, on both rough-surfaced and smooth-surface types, and on the nuclear envelope. The localization of this enzyme was identical with those described by Tice & Barneett (1962) and Ericsson (1966) in liver cells, and by Kanamura (1975) in the tracheal epithelium. We owed this distinct demonstration, at least in part, to suggestions of Tice & Barneett (1962) and Hugon et al. (1970) to use sucrose for physiological osmolarity.
In this kind of investigation, an undecided question concerns the specificity of the various phosphatases (specific and non-specific) demonstrated histochemically. Since both non-specific acid phosphatase and alkaline phosphatase are capable of hydrolyzing G6P, and G-6-Pase may split glycerophosphate at even a low rate, it is obviously necessary to produce some proof of histochemical specificity. In our control preparations, where G6P was omitted from the incubating medium to establish substrate specificity, or where G6P was replaced by glycerophosphate in the medium, no enzyme activity was shown. Wachstein and Meisel (1957) reported that livers of man, rabbit and guinea pig revealed almost no non-specific activity in a substrate mixture (pH 7.2) in which G6P was replaced by glycerophosphate, but some activity was shown in the liver of dog, rat, and mouse, when the incubation time was prolonged. It is well known that the pH optimum is 6.5~6.7 for G-6-Pase, as contrasted to 5.0 for non-specific acid phosphatase and 9.4 for alkaline phosphatase (Burstone 1962, Pearse 1968) and that incubation (at 37°C) in acetate buffer at pH 5.0 results in rapid irreversible inactivation of the enzyme (Tice & Barrnett 1962, Lillie 1965). Furthermore, Tice & Barrnett (1962) and Manns (1968) reported that over the pH range 6.5~7.0 no activity was present if fructose-6-phosphate, ribose-5-phosphate, glucose-1-phosphate, or fructose-1,6-di-phosphate were used in place of G6P.

Pearse (1968) stated that formalin destroyed G-6-Pase but not acid or alkaline phosphatase and showed that the absence of any reaction with G6P with sections prefixed in formalin was sufficient proof of the specificity of the reaction. Thus we used the unfixed frozen sections and then post-fixed them after incubation with formalin for light microscopy or with osmic acid for electron microscopy. However, Tice & Barrnett (1962) demonstrated enzyme activity in liver tissues following prefixation in hydroxyadipaldehyde. Ericsson (1966) perfused the livers with glutaraldehyde. Manns (1968) fixed fresh frozen sections in neutral formalin prior to incubation and no detectable loss in activity was shown by prefixation for up to 10 minutes. Hugon et al. (1970) prefixed the jejunal epithelium in glutaraldehyde. Kanamura (1975) tried to prefix the tracheal epithelium of rats in glutaraldehyde for electron microscopic demonstration of G-6-Pase activity with distinct localization of enzyme activity.

Tice & Barrnett (1962) discussed the exact localization of G-6-Pase in the rough-surfaced and smooth-surfaced endoplasmic reticulum, glycogen areas, and nuclear envelope. With respect to rough-surfaced endoplasmic reticulum, they described how in fresh preparations the deposits of the final product occurred for the most part on the limiting membrane of the cisternae or just outside the membrane and that occasional deposits occurred just outside the membrane. The investigators suggested that the occasional outside localization had some appeal because of the hyaloplasmic position of rat liver glycogen (Porter & Bruni 1959) and that the endoplasmic reticulum could be concerned with the transport of glucose from glycogen areas because the structure would be an intracellular transport system of tubules. They expected the localization of final product in some elements of the smooth-surfaced endoplasmic reticulum since there were connections between smooth- and rough-surfaced endoplasmic reticulum in liver cells and profiles of the smooth-surfaced variety commonly surrounded glycogen areas. In view
of the enzyme found within the nuclear envelope, they suggested the non-artifactual nature of this activity by the fact that deposits occurred only in the nuclear envelope of hepatic cells and that high levels of G-6-Pase activity had been found to occur in relatively clean nuclear fractions prepared from hepatic cells. They postulated that the enzyme activity within the nuclear membrane clearly related this structure to the enzymatically active endoplasmic reticulum and that there would be morphological continuity between the two structures (Watson 1955).

From the present investigation, it is suggested that better ultrastructural visualization of G-6-Pase activity in liver cells can be obtained by using unfixed frozen sections incubated with modifications (B,C) of Wachstein-Meisel medium.

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Fig. 1. Glucose-6-phosphatase activity, mouse liver cells, unfixed frozen section incubated in the medium A (modification of Chi-quoine medium). Heavy deposits of final product are observable in hepatocytes of periportal fields. ×100.

Fig. 2. A section incubated in the medium-B (the first modification of Wachstein-Meisel medium). Final product is more abundant in periportal zones than in centrilobular zones. Fine granules are distributed throughout the cytoplasm of hepatocytes, but no reaction product is present in nuclei. ×100.

Fig. 3. A section incubated in the medium-C (the second modification of Wachstein-Meisel medium). The enzyme activity is the same as in fig.2 in the amount and localization of the reaction product. ×100.
Fig. 4. Unfixed frozen section incubated in the medium A. Coarse granular deposits of reaction product occur on cisternae between mitochondria (M) which are unreactive. Some deposits are observable on the unclear envelope. ×22500.

Fig. 5. A section incubated in the medium B. Finer deposits granules of final product are deposited on the membranes of rough-and smooth-surfaced (v) endoplasmic reticulum and nuclear envelope. Occasional deposits are within cisternae and nucleus, and outside the cisternal membranes. ×22500.
Fig. 6. Other section incubated in the same medium as that of figure 5. L: lipid. ×22500.

Fig. 7. A section incubated in the medium-C.
Granular deposits of final product occur on the dilated membranes of dilated cisternae between mitochondria (M) which are non-reactive and somewhat disfigured, and on the nuclear envelope. Occasional deposits are scattered among the chromatin. ×22500.
Fig. 8. Other section treated as figure 7.
Reactive granules are found on dilated profiles of rough-surfaced endoplasmic reticulum and smooth-surfaced vesicles (v). Gl: glycogen area, J: cell junction. × 22500.