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HUMAN LIVER GLUCOSE-6-PHOSPHATASE CHARACTERISTICS OF THE DETERGENT-SOLUBILIZED ENZYME

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SUMMARY

Detergent-solubilized human liver glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) has been found to have inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) and pyrophosphate-glucose phosphotransferase activity (not yet classified). It is composed primarily of lipid and protein, with a small amount of RNA, and trace amounts of calcium and magnesium. Pigs and rabbits were immunized with it, and the antiserum also reacted with similar preparations from human kidney and intestine, and also from monkey and guinea pig liver. Liver from a child with glycogen storage disease, type I, did not produce a precipitin line in agar when it was diffused into the antiserum.

INTRODUCTION

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) is a microsomal enzyme found primarily in liver, kidney and intestine[1]. The rat liver enzyme can be induced by cortisol administration, fasting and alloxan diabetes, and suppressed by insulin[2] and growth hormone[3] administration. Human liver glucose-6-phosphatase decreases with aging and is reduced in children with leukemia[4]. Liver and intestinal glucose-6-phosphatase and inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) are reduced or absent in children with von Gierke's disease[5]. Studies with rat liver glucose-6-phosphatase have indicated that this enzyme may also have inorganic pyrophosphatase activity[6-8], and these two activities have been found to be directly proportional in liver from children[3].

This report describes several additional characteristics of detergent-solubilized human liver glucose-6-phosphatase.

MATERIALS AND METHODS

Human liver was obtained at autopsy, placed in ice-cold buffer, and either homogenized immediately or wrapped in cellophane and frozen at −20°. The 10%

homogenates were made in a solution containing 0.12 M KCl, 0.005M $MgCl_2$, and 0.02 M Tris-HCl (pH 7.5); and also in 0.25 M sucrose in distilled water. Livers were screened for activity by homogenizing 2 g with a glass-teflon homogenizer at 600 rev./min and then assaying the homogenate. The livers with suitable activity were used for subsequent studies. 60 g of liver were homogenized for 10 min with a Waring blender at high speed in 600 ml buffer. This was done in a cold room, and for all subsequent steps the enzyme was also kept at 0°.

The fraction containing mitochondria and microsomes was prepared by centrifuging the homogenate at 1000 rev./min for 10 min, discarding the residue, and then centrifuging at 40 000 rev./min in a No 40 Spinco rotor for 10 min in a refrigerated ultracentrifuge. Several specimens were centrifuged again for 60 min and additional enzyme was not sedimented. This residue was resuspended to one-sixth of the original volume in buffer containing 5% Triton X-100.

The 5% Triton X-100 suspension was centrifuged at 40 000 rev./min for 10 min. Saturated ammonium sulfate (adjusted to pH 7.5 with NH_4OH) was added to the supernatant to make a final concentration of 60%. The suspension was centrifuged at 10 000 rev./min for 10 min, and the precipitate resuspended in buffer or 0.25 M sucrose at 10–20 mg protein/ml.

A 5-ml aliquot of this suspension was then eluted with buffer from a 2.5 cm × 40 cm Sephadex G-100 column in 1.5-ml fractions. The fractions were checked for absorbance at 280 mμ, and were assayed for glucose-6-phosphatase and inorganic pyrophosphatase activity. Mixtures composed of 1.0 ml of the 60% ammonium sulfate precipitate suspended in 0.25 M sucrose and 0.1 ml of [^{14}C]glucose 6-phosphate (3.5 mC/mmole, 0.05 mC/ml) or $^{32}PP_i$ (356 mC/mmole, 0.74 mC/ml) were prepared. These mixtures were incubated at 0° for 30 min and then were eluted with buffer from 2 cm × 15 cm Sephadex G-100 columns in 1.5-ml fractions. The first of two radioactivity peaks coincided with the glucose-6-phosphatase and pyrophosphatase peak. From the counts/min associated with the enzyme peak, the quantity of substrate bound to the enzyme was calculated and is summarized in Table III.

Assays for these enzymes were proportional to time and enzyme concentration. Glucose-6-phosphatase was assayed by mixing 0.1 ml of the enzyme suspension (5–15 mg protein/ml), 0.1 ml glucose 6-phosphate (0.1 M), and 0.3 ml of 0.1 M maleate buffer (pH 6.5). This reaction mixture was incubated at 37° for exactly 15 min, then chilled in ice and 0.25 ml of 13% trichloroacetic acid was added. An additional mixture which was kept at 0° was the control. All assays were done in duplicate and the activity was calculated as the difference in inorganic phosphorus between the incubated and the control mixture, as mμmoles phosphorus/mg protein per min.

Pyrophosphatase was assayed by the same method, except the reaction mixture contained 0.1 ml of 0.1 M sodium pyrophosphate adjusted to pH 5.8 with acetic acid, 0.1 ml of the enzyme suspension and 0.3 ml of 0.1 M acetate buffer (pH 5.8).

Phosphotransferase assays were done by incubating 0.1 ml of 0.01 M pyrophosphate at pH 5.2, 0.1 ml of 0.05 M D-glucose, 0.1 ml enzyme suspension and 0.2 ml of 0.05 M sodium acetate (pH 5.2), at 37° for 15 min. The reaction was terminated by addition of 0.25 ml 10% perchloric acid. After adjusting this mixture to pH 7.0 with 6 M KOH, and centrifuging at 5000 rev./min for 10 min, the glucose 6-phosphate in the supernatant was determined spectrophotometrically with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ as summarized by NORDLIE AND ARION[6].

NADH oxidase (EC 1.6.99.3) and succinate dehydrogenase (EC 1.3.99.1) were assayed using a Yellow Springs Instrument Company oxygen polarograph. They were run at two different enzyme concentrations at 37°, and the activities were calculated as the average of the two assays. The reaction mixture for NADH oxidase contained 0.5 mmole NADH, 0.02 ml of 1% cytochrome *c*, and 0.02 M Tris-HCl (pH 7.5) in 3 ml water. Reactions were started by addition of 0.1–0.5 ml enzyme. The succinate dehydrogenase assay was the same except 0.05 ml of 1 M succinate solution was substituted for NADH and cytochrome *c* was not added.

A rabbit and a pig were immunized with the ammonium sulfate precipitate resuspended in 0.25 M sucrose. During 10 weeks each animal received 20 injections composed of 1 ml of enzyme suspension diluted with 1 ml of incomplete Freund's adjuvant. Agar diffusion studies were done in 2% agar which contained 0.001% phenol. Immunoelectrophoresis was done using agarcoated slides. Antiserum inhibition studies were done by preincubating the enzyme preparations at 0° for 48 h with the pig antiserum and also with normal pig serum as the control.

K_m was determined for two or more samples of each fraction using initial reaction rates by the method of LINEWEAVER AND BURK[11]. Substrate concentrations for the K_m determinations were 0.1, 0.05, 0.025, 0.013, and 0.006 M, and the enzyme was suspended in 0.25 M sucrose. The pH optima were determined at 37° by preparing duplicate assay mixtures at various pH's, and with 15-min incubation times for the assays. One of them was assayed and the other was diluted to 5 ml with distilled water for pH determination.

Total lipid was determined by evaporating a 2-ml aliquot to dryness in a tared beaker, and weighing it again. Then it was extracted with five successive 2-ml aliquots of chloroform–methanol (1:1, by vol.), filtered, and the extract was evaporated to dryness and weighed.

Magnesium and calcium were determined by atomic absorption spectrophotometry. Protein and RNA were determined by the methods of LOWRY *et al.*[9] and CERIOTTI[10].

Glucose 6-phosphate, disodium, β-NADH, horse heart cytochrome *c* (Type III), and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company. [^{14}C]glucose 6-phosphate and sodium [^{32}P]pyrophosphate were obtained from Nuclear Chicago Corporation.

TABLE I

HUMAN LIVER ENZYME

Activity in mμmoles substrate/mg protein per min.

	Glc-6-P	*PP_i*	*Phospho-transferase*	*NADH oxidase*	*Succinate dehydro-genase*
Liver homogenate					
Mean activity	15.6	24.0	15.6	0.02	0.007
Number of samples	13	13	4	11	9
60% ammonium sulfate precipitate					
Mean activity	5.2	23.2	12.8	0	0
Number of samples	5	5	4	4	4

RESULTS

Table I summarizes data which compare the activity of the detergent-solubilized enzyme preparation with 10% liver homogenate. The three microsomal activities were solubilized and the mitochondrial activities were not. There was a tendency for less glucose-6-phosphatase activity to be found in the solubilized preparation when compared to the other two microsomal activities.

TABLE II

PROPERTIES OF HUMAN LIVER GLUCOSE-6-PHOSPHATASE AND INORGANIC PYROPHOSPHATASE

	pH optimum	K_m *(M)*
10% homogenate		
Glc-6-*P*	6.5	0.0066
PP_i	5.8 6.5	0.0025
5% Triton X-100 supernatant		
Glc-6-*P*	6.5	0.0080
PP_i	5.8	0.0026

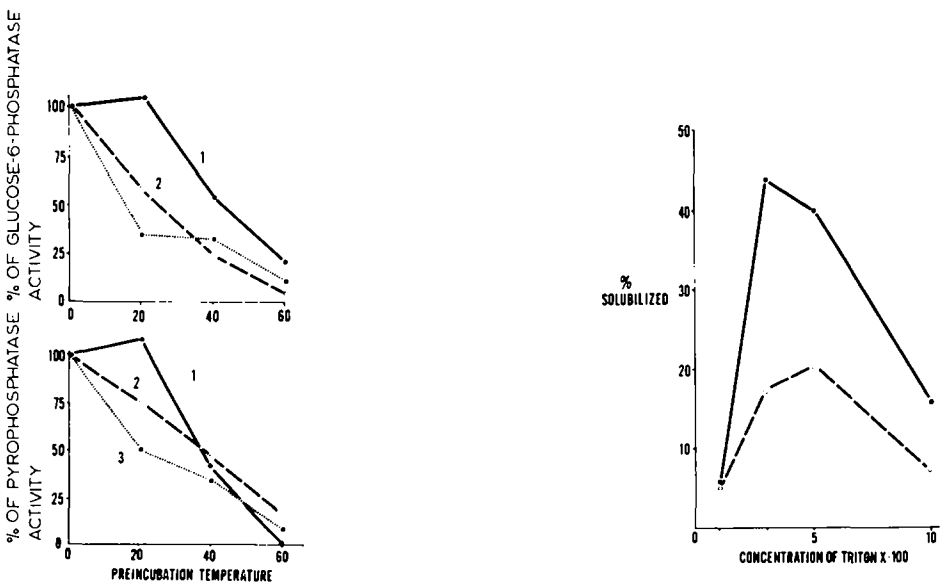


Fig. 1. 1, Human liver homogenate; 2, 40 000 rev./min residue; and 3, 5% Triton supernatant were preincubated for 30 min at different temperatures. Glucose-6-phosphatase and inorganic pyrophosphatase activity were determined.

Fig. 2. The 40 000 rev./min residue of human liver homogenate was resuspended in different concentrations of Triton X-100 and then centrifuged again at 40 000 rev./min. The supernatant was assayed for glucose-6-phosphatase (○— – ○) and inorganic pyrophosphatase (●—●). The data represent the per cent of each enzyme which remained in the 5% Triton X-100 supernatant in similar experiments with 7 different livers.

The pH optimum and the K_m of these activities were determined before and after solubilization with Triton X-100. The data in Table II summarize the findings, which indicate that the K_m and pH optimum was essentially the same for the original 10% homogenate and the detergent-solubilized enzyme.

The effect of preincubation for 30 min at various temperatures is summarized in Fig. 1. Glucose-6-phosphatase and inorganic pyrophosphatase were reduced proportionately as the preincubation temperature increased, and the more purified preparations were more sensitive to heat inactivation. Pork insulin (1 unit/ml) was added to additional aliquots of liver homogenate and did not alter the results of preincubation at different temperatures.

When the 40 000 rev./min residue was suspended in 5% Triton X-100 in buffer and allowed to stand for 0, 30, 60 and 120 min at 0° before centrifugation, there was no increase in the solubilization of either enzyme activity. Several concentrations of Triton X-100 and deoxycholate were used to solubilize these activities. The data in Fig. 2 indicate that 3–5% Triton X-100 was the optimal concentration for solubilizing both. Several deoxycholate concentrations were tried (0.1%, 0.25%, 0.5%, 1.0%, 2.0%) and 0.5% was found to be the optimal concentration for both enzyme activities. Deoxycholate was similar to the Triton X-100 and solubilized less glucose-6-phosphatase than pyrophosphatase.

Data from one of the Sephadex G-100 columns is summarized in Fig. 3. The peak

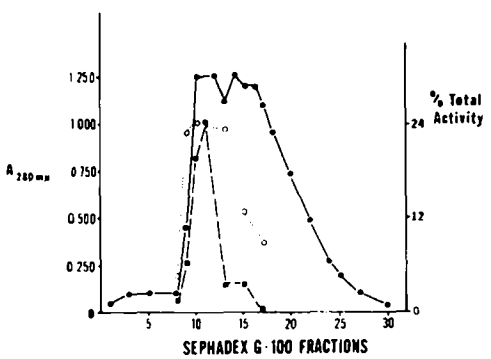


Fig. 3. Data are from one of several similar experiments. Solubilized (5% Triton X-100) human liver glucose-6-phosphatase and inorganic pyrophosphatase were eluted from a Sephadex G-100 column in 1.5-ml fractions. The absorbance at 280 mμ (●—●) was determined after diluting (1:5) an aliquot of each fraction with distilled water. Glucose-6-phosphatase (■— –■) and inorganic pyrophosphatase (○ · · · · ○) activities were determined with aliquots of each fraction, and their recovery in different fractions is plotted as a per cent of the total recovery.

activity for both glucose-6-phosphatase and pyrophosphatase were found in the same fractions.

The composition of the three most active fractions from the Sephadex columns is summarized in Table III. It contained 38% lipid, 20.9 mg RNA/g protein and small amounts of magnesium and calcium. Magnesium was not included in any preparative solution when the fractions were to be analyzed for it. When added to the ammonium sulfate suspension, small amounts of [^{14}C]glucose 6-phosphate and $^{32}PP_i$ were associated with the enzyme peak from the Sephadex G-100 columns. These columns

TABLE III

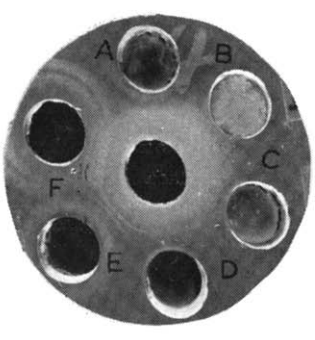
COMPOSITION OF ENZYME AFTER SEPHADEX G-100 COLUMN*

	Mean	*Range*
Glucose-6-phosphatase activity (mμmoles/mg per min)	2.4	0.8 – 4.4
Inorganic pyrophosphatase activity (mμmoles/mg per min)	16.0	8.4 – 34.4
Total lipid (% dry weight)	38.0	17.0 – 55.0
Ribonucleic acid (mg/g protein)	20.9	8 – 44
Magnesium (μmoles Mg/g protein)	8.2	7 – 9
Calcium (μmoles Ca/g protein)	26.0	14 – 36
[^{14}C] Glucose 6-phosphate (μmoles/g protein)	7.5	5.6 – 11.0**
$^{32}PP_i$ (μmoles/g protein)	119.0	65 – 173**

* Data are the mean of preparations from 7 different livers.
** Mean of experiments with 3 different livers.

were eluted at 0° in order to reduce the conversions of these substrates to [^{14}C]glucose and $^{32}P_i$, respectively.

The pig and rabbit antisera were studied for their organ and species specificity. Figs. 4 and 5 are photographs of agar double-diffusion studies which show that the pig antiserum contained precipitating antibodies for human kidney and intestine glucose-6-phosphatase preparations and also for monkey and guinea pig liver. The pig antiserum always produced a precipitin line when diffused against the resuspended normal human liver ammonium sulfate precipitate. Addition of 0.01 M glucose 6-phosphate and inorganic pyrophosphate to the agar had no effect on the development of precipitin lines. Fig. 6 shows that liver from a child with glycogenstorage disease, type I, failed to produce a precipitin line with the pig antiserum. Fig. 7 shows that only a single



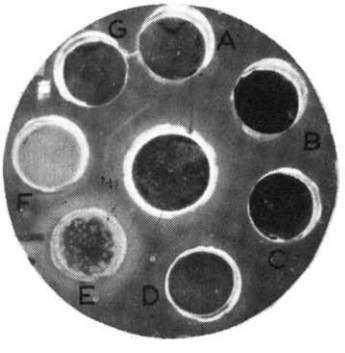


Fig. 4. Agar double-diffusion study showing a precipitin line between pig anti-human-liver (ammonium-sulfate precipitate) serum in the center and: A, resuspended (10 mg/ml) human liver ammonium sulfate precipitate; E, resuspended (10 mg/ml) human kidney ammonium sulfate precipitate; F, resuspended (10 mg/ml) human intestine ammonium sulfate precipitate; and no precipitin line for B, pig liver homogenate (10 mg/ml); C, resuspended (10 mg/ml) pig liver ammonium sulfate precipitate; D, human liver homogenate (10 mg/ml).

Fig. 5. Agar double-diffusion study showing precipitin line between pig anti-human-liver (ammonium sulfate precipitate) serum in center and resuspended (10 mg/ml) ammonium sulfate precipitates from liver of B, guinea pig; F, monkey; G, human; and no precipitin lines for A, dog; C, pig; D, rat and E, rabbit.

TABLE IV

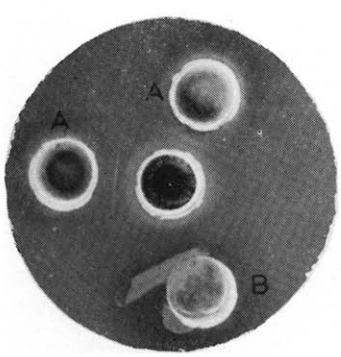
ANTISERUM INHIBITION STUDIES. % OF CONTROL ACTIVITY

	Glc-6-P (%)	PP_i (%)
Human liver homogenate (5)	106	116
Solubilized enzymes (3)	39	54

precipitating antibody was detected in the rabbit antiserum during immunoelectrophoresis, and this was also true for the pig antiserum.

Experiments were done to determine whether the pig antiserum would inhibit the enzyme activities. Addition of 0.1 ml of antiserum to the assay mixture did not cause significant inhibition. When liver homogenate was preincubated with the antiserum, no significant inhibition was found. Preincubation with the liver ammonium sulfate precipitate did reduce glucose-6-phosphatase and pyrophosphatase to 39% and 54% of the control values (Table IV).

The precipitating antibodies in the pig antiserum were precipitated by addition of solid ammonium sulfate. Most of the antibody was found in the fraction that precipitated between 34% and 40% ammonium sulfate. This fraction was resuspended in 0.25 M sucrose at 20 mg protein/ml and was found to have concentrated the pre-



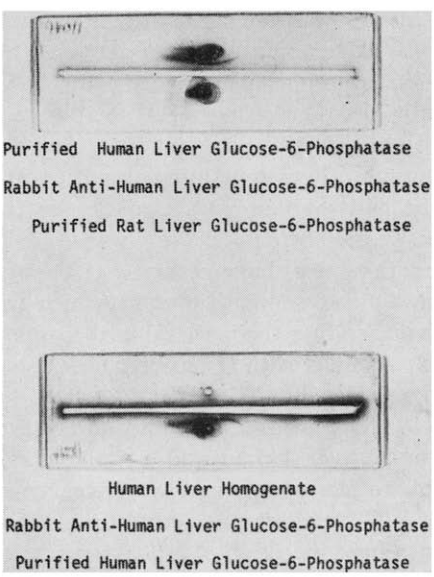


Fig. 6. Agar double-diffusion study showing precipitin lines between pig anti-human-liver (ammoniumsulfate precipitate) serum in center and 2 resuspended (10 mg/ml) normal human liver ammonium sulfate precipitates (A). There is no precipitin line for the other human liver ammonium sulfate precipitate prepared from a child with type 1 glycogen storage disease (B).

Fig. 7. Immunoelectrophoresis slides showing single precipitin line between the rabbit antiserum and the resuspended (10 mg/ml) human liver ammonium sulfate precipitate at top and bottom, and absence of line for the human liver homogenate (10 mg/ml) (bottom) and resuspended rat liver ammonium sulfate precipitate (top).

cipitating antibody 2-3 fold. This concentrated antiserum still did not inhibit the enzyme activity of liver homogenate.

DISCUSSION

The effect of using autopsy liver on the activities of these enzymes has been studied previously[4], and there was insignificant change in enzyme activity during the first 24 h after death. After homogenization, or during prolonged storage at −20°, both activities decline steadily. These studies were done primarily with adult human liver, which has previously been shown to be 5-10-fold less active than during the first year or two of life[4].

Solubilization of enzyme activities from mitochondrial or microsomal particles by detergents is usually determined by the fact that the activity in question no longer sediments during ultracentrifugation under conditions where it previously did sediment. This detergent solubilization can result in truly soluble enzyme proteins, or it can simply reduce the size of the colloidal particles so that they no longer sediment during ultracentrifugation. The analyses of the detergent-solubilized enzymes in this report indicate that they contain 38% lipid and very small amounts of RNA. This suggests that this particular "solubilization" removed most of the RNA and dispersed the large lipoprotein colloidal particles into smaller ones. NORDLIE AND ARION[6] mentioned that detergent-solubilized glucose-6-phosphatase from rat liver had a tendency to aggregate during storage, and they suggested that for rat liver the detergents were probably also dispersing large microsomal particles into small ones.

Magnesium does not activate the microsomal glucose-6-phosphatase and inorganic pyrophosphatase from human liver, but does tend to stabilize them during storage and heating[3]. The small amounts of calcium and magnesium found in the column fractions suggest that they are contaminants. The unexpected coincidence between the magnesium content and the quantity of glucose 6-phosphate bound by the active column fractions leaves the question of whether the enzyme contains magnesium still not definitely answered. The binding of substrate by the active column fractions may have occurred at the active site or at non-specific sites. It may have been associated with proteins which are not part of the enzyme. The only additional evidence that the material in the column fractions was homogeneous comes from the experiments with the antiserum. A single precipitin line was found during immunoelectrophoresis.

The antiserum studies indicate that there are antigenic similarities between human liver, kidney and intestine, and monkey and guinea pig liver. The absence of this antigen in a patient with glycogen-storage disease and the ability of the antiserum to inhibit the enzyme activity provide evidence that this antigen is closely associated with liver glucose-6-phosphatase.

A recent report[13] by NORDLIE AND SNOKE has presented data which indicate that the detergents eliminate the difference in rat liver glucose-6-phosphatase activity which results from cortisol administration.

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