- enzyme replacement studies. *Pediat. Res.*, 9: 313 (1975).
6. Glaser, J. H., McAlister, W. H., and Sly, W. S.: Genetic heterogeneity in multiple lysosomal hydrolase deficiency. *J. Pediat.*, 85: 192 (1974).
 7. Glaser, J. H., Roozen, K. J., Brot, F. E., and Sly, W. S.: Multiple isoelectric and recognition forms of human β -glucuronidase activity. *Arch. Biochem. Biophys.*, 166: 536 (1975).
 8. Glaser, J. H., and Sly, W. S.: β -Glucuronidase deficiency mucopolysaccharidosis: Methods for enzymatic diagnosis. *J. Lab. Clin. Med.*, 82: 969 (1973).
 9. Hall, C. W., Cantz, M., and Neufeld, E. F.: A β -glucuronidase deficiency mucopolysaccharidosis: Studies in cultured fibroblasts. *Arch. Biochem. Biophys.*, 155: 32 (1973).
 10. Hickman, S., and Neufeld, E. F.: A hypothesis for I-cell disease: Defective hydrolases that do not enter lysosomes. *Biochem. Biophys. Res. Commun.*, 49: 992 (1972).
 11. Jolly, R. D.: Animal model of human disease: Mannosidosis. *Amer. J. Pathol.*, 74: 211 (1974).
 12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265 (1951).
 13. Neufeld, E. F., Lim, T. W., and Shapiro, L. J.: Inherited disorders of lysosomal metabolism. *Ann. Rev. Biochem.*, 44: 357 (1975).
 14. Sly, W. S., Glaser, J. H., Roozen, K., Brot, F. E., and Stahl, P.: Enzyme replacement studies with β -glucuronidase deficiency. In: J. M. Tager, G. J. M. Hooghwinkel, and W. T. Daems: *Diseases, Enzyme Therapy in Lysosomal Storage*, pp. 288-291 (North-Holland, Amsterdam, 1974).
 15. Sly, W. S., Quinton, B. A., McAlister, W. H., and Rimoin, D. L.: Beta-glucuronidase deficiency: Report of clinical, radiologic and biochemical features of a new mucopolysaccharidosis. *J. Pediat.*, 82: 249 (1973).
 16. Suzuki, K., Suzuki, Y., and Fletcher, T. F.: Further studies on galactocerebroside β -galactosidase in globoid cell leukodystrophy. *Advan. Exp. Med. Biol.*, 19: 487 (1972).
 17. Thorpe, S. R., Fiddler, M. B., and Desnick, R. J.: Enzyme therapy. IV. A method for determining the *in vivo* fate of bovine β -glucuronidase in β -glucuronidase deficient mice. *Biochem. Biophys. Res. Commun.*, 61: 1464 (1974).
 18. Thorpe, S. R., Fiddler, M. B., and Desnick, R. J.: Enzyme therapy. V. *In vivo* fate of erythrocyte-entrapped β -glucuronidase in β -glucuronidase-deficient mice. *Pediat. Res.*, 9: 918 (1975).
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Enzyme replacement lysosomes
enzyme uptake storage disease
 β -glucuronidase

Human β -Glucuronidase. II. Fate of Infused Human Placental β -Glucuronidase in the Rat

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Summary

Human placental β -glucuronidase could be identified in rat organs by heating organ extracts to 65° which selectively inactivated endogenous rat enzyme. Infused enzyme was rapidly cleared from rat plasma ($t_{0.5}$ of 3.5 min). From 50-60% of the infused dose was accounted for in rat liver and spleen 24 hr after infusion. Removal of abdominal viscera, including the spleen, and interruption of the portal circulation before infusion slowed the plasma disappearance ($t_{0.5}$ of 60 min) and allowed significant uptake by bone and other organs. Subcellular fractionation of liver 18 hr postinfusion localized the human enzyme in the mitochondrial-lysosomal fraction. The half-disappearance times of infused human enzyme were 2.6 days in rat liver and 5.8 days in rat spleen. Periodate treatment of human placental β -glucuronidase destroyed 90% of its binding to concanavalin A-Sepharose, reduced its heat stability, and abolished its rapid clearance from rat plasma after infusion.

Speculation

Experiments with the rat model for evaluation of the uptake, distribution, intracellular localization, and metabolic fate of infused human β -glucuronidase can provide a number of answers about enzyme replacement therapy that could not be obtained

easily by human experimentation. The experiments presented suggest that placental β -glucuronidase is cleared predominantly by organs with significant reticuloendothelial components. The clearance mechanism appears to recognize the carbohydrate structure of the enzyme, but may recognize carbohydrate components different from those responsible for enzyme uptake by fibroblasts. Delineation of the cell-specific receptors responsible for organ-specific localization of native and modified human enzymes such as β -glucuronidase may be important to developing a rational basis for enzyme replacement therapy in lysosomal storage disease.

Enzyme replacement therapy for lysosomal storage disorders due to single enzyme deficiency diseases was initially suggested by Hers (13). Interest in enzyme replacement (see reviews, References 2 and 8) was greatly stimulated by the discoveries of Neufeld and coworkers (21) of biochemical correction of cultured fibroblasts *in vitro* by lysosomal enzymes. For a number of reasons, outlined elsewhere (23), β -glucuronidase deficiency mucopolysaccharidosis seemed attractive as an experimental model system to test the feasibility of enzyme replacement in man. However, an animal model system seemed desirable to answer questions about the uptake, fate, and distribution of infused human enzyme that could not be answered in man.

Studies with animal fibroblasts indicated that human β -glucuronidase, identifiable by its thermostability, was recognized and taken up by fibroblasts from a number of animal species (9). The heat stability of human β -glucuronidase also allows it to be identified in extracts of animal organs. Thus, even though there was no animal model for human β -glucuronidase deficiency mucopolysaccharidosis, the ability to identify infused human β -glucuronidase in animal tissues by its heat stability suggested that many questions about lysosomal enzyme replacement which could not be answered by human experimentation might be answered by studying the fate of infused human enzyme in certain experimental animals. In this paper, we examine the uptake, tissue distribution, intracellular localization, and fate of infused human placental β -glucuronidase in whole rats. A preliminary report of this work was presented previously (1).

MATERIALS AND METHODS

ISOLATION OF HUMAN PLACENTAL β -GLUCURONIDASE

β -Glucuronidase activity was solubilized from tissues as described by Brot *et al.* (5). Frozen placenta (20 g) was homogenized in a Waring Blender in 5 volumes of 0.10 M Tris-HCl, pH 8.0, 0.15 M NaCl. A $2,500 \times g$ supernatant of this crude homogenate was adsorbed batchwise to antibody-Sepharose (Sepharose beads linked to goat anti-rat preputial β -glucuronidase antibody). The beads were washed with 0.15 M NaCl to remove nonspecifically adsorbed protein, and then β -glucuronidase activity was eluted with 6 M urea. The eluate from the affinity column was made 3 M urea, 0.01 M sodium phosphate, pH 5.8, and was passed over a CM-Sephadex column. The column was washed with 0.01 M sodium phosphate, pH 5.8, and β -glucuronidase activity was eluted with 0.2 M sodium phosphate, pH 7.0 (10). The specific activity of the 1,000-fold purified enzyme preparation was 300,000 units/mg.

ANIMAL PREPARATIONS

Male Sprague-Dawley rats were used for infusion and subcellular localization experiments. The animals were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). The femoral artery and contralateral vein were cannulated with PE10 tubing filled with heparinized saline. Enzyme preparations in 0.15 M NaCl were infused using a Harvard constant infusion pump. Arterial blood samples were taken, starting 20 sec after termination of the enzyme infusion, using heparinized capillary tubes. Plasma taken from each capillary tube was used for enzyme assays. For organ localization experiments the cannulated vessels were tied off, wound clips were applied to the incisions, and animals were killed by decapitation at the times indicated.

ASSAY OF ORGAN β -GLUCURONIDASE ACTIVITY

Animals were killed by decapitation and organs were removed, weighed, and placed on ice. Tissues were homogenized (1 g) in 7 ml 0.02 M Tris-HCl, pH 7.5, 0.075 M NaCl in a Polytron homogenizer for 30 sec. This crude homogenate was diluted further (1:5) in 0.02 M Tris-HCl, pH 7.5, 0.075 M NaCl, bovine serum albumin, 5 mg/ml, and 0.2% sodium deoxycholate. Endogenous β -glucuronidase activity was inactivated by incubation of dilute crude homogenate in a 65° water bath for 90 min. These homogenates were then assayed for β -glucuronidase activity using 10mM 4-methylumbelliferyl- β -D-glucuronide as described previously (9, 11). One unit of enzyme activity is defined as the activity which releases 1 nmol 4-methylumbelliferone/hr.

SUBCELLULAR FRACTIONATION OF RAT LIVERS

Subcellular fractionation was performed by a modification of the method of De Duve *et al.* (7). Sprague-Dawley rats weighing 170 g were killed by decapitation and the liver was rapidly removed, weighed, cut into small pieces, and suspended in 0.25 M sucrose, 1mM EDTA, 0.02 M Tris-HCl, pH 7.4 (4 ml/g

tissue), and homogenized for 15 sec at 1,200 rpm in a Potter-Elvehjem homogenizer with a Teflon pestle. The resulting slurry was centrifuged at $480 \times g$ for 10 min in a Sorvall 55-34 rotor. The pellet was twice homogenized and recentrifuged ($270 \times g$, 10 min) and the three supernatants were pooled, yielding a crude extract (CE), which was centrifuged at $25,000 \times g$ for 10 min. The resulting mitochondrial-lysosomal (ML) pellet was resuspended in 2 volumes of 0.25 M sucrose, 1 mM EDTA, 0.02 M Tris-HCl pH 7.4, homogenized at 700 rpm for 10 sec, and recentrifuged at $25,000 \times g$ for 10 min. The washed ML Pellet was resuspended (6 ml/g liver). The two preceding supernatants were combined and centrifuged at $105,000 \times g$ for 35 min in a 50 Ti rotor of a Beckman L2-65B ultracentrifuge. The resulting supernatant corresponds to the cytosol fractions, and the final microsomal pellet (P) was resuspended (6 ml/g liver).

Values for the original homogenate were obtained by combining data from cytoplasmic extract (CE) and nuclear fraction (N) as described by DeDuve *et al.* (7).

PERIODATE TREATMENT OF HUMAN PLACENTAL β -GLUCURONIDASE

β -Glucuronidase activity was treated with sodium metaperiodate by the method of Spiro (24), in which 1.73×10^6 units of placental β -glucuronidase activity (specific activity 300,000 units/mg protein) in 0.05 M sodium acetate buffer, pH 4.5, was incubated with sodium metaperiodate (final concentration 0.02 M) at 4° in the dark for 5 hr. The reaction was stopped by the addition of 0.1 volume ethylene glycol (final concentration 1.63 M), and the sample was dialyzed overnight against 4 liters 0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl. A control sample was treated in the same manner except for addition of sodium metaperiodate. Recovery of initial enzyme activity after dialysis was 86% in the periodate-treated sample and 89% in the control.

RESULTS

Previous work demonstrated that added human platelet β -glucuronidase could be identified in homogenates of rat organs by its stability to thermal inactivation conditions which inactivate the rat endogenous β -glucuronidase (9). These experiments led us to predict that infused human enzyme could be similarly identified in rat organs after infusion. The experiments presented in Figure 1 demonstrate that heat-stable β -glucuronidase

Fig. 1. Heat stability of human β -glucuronidase activity in rat liver and spleen after infusion. Two 250-g rats received either 250,000 units human placental β -glucuronidase in 1.0 ml 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, or 1.0 ml of Tris-buffered saline without enzyme (saline-infused control). Homogenates of liver and spleen were prepared from animals killed 24 hr after the infusions, and the residual β -glucuronidase activity was measured after preincubation of the homogenates at 65° for varying times. ●: β -glucuronidase activities in liver and spleen from the animal which received human β -glucuronidase; ▲: β -glucuronidase activities in organs from the saline-infused control animal.

Table 1. Rat β -glucuronidase and added human placental β -glucuronidase remaining after heating organ homogenates¹

Tissue	No human enzyme added	Heat-stable β -glucuronidase recovered, units/g					
		41,589 units/g human enzyme		4,216 units/g human enzyme		451 units/g human enzyme	
		units/g	% of added activity	units/g	% of added activity	units/g	% of added activity
Liver	24	37,504	90.2	4,136	98.1	432	95.8
Spleen	8	37,504	90.2	3,680	87.3	432	95.8
Bone	0	39,424	94.8	3,872	91.8	320	71.0
Kidney	0	37,504	90.2	3,824	90.7	400	88.8
Lung	0	39,424	94.8	3,968	94.1	368	81.7
Skin	0	39,424	94.8	3,824	90.7	448	99.4
Heart	0	37,504	90.2	3,872	91.8	384	85.2
Brain	8	38,144	91.7	4,416	104.7	384	85.2
Muscle	0	41,344	99.4	3,872	91.8	416	92.3

¹ Organs were homogenized and diluted 1:5 as described in "Materials and Methods." One-milliliter aliquots were heated for 90 min at 65° with or without addition of human placental β -glucuronidase before heating. The organ content of rat β -glucuronidase was measured for each homogenate before addition of human enzyme or heating. Levels of rat β -glucuronidase before heating, in units per g, were: liver, 130,240; spleen, 90,240; bone, 34,469; kidney, 22,944; lung, 21,504; skin, 3,637; heart, 3,424; brain, 3,312; and muscle, 2,400.

Fig. 2. Plasma levels of β -glucuronidase activity in a β -glucuronidase-infused and control rat. These results were obtained from two animal infusions exactly as described in the legend to Figure 1. Blood samples were taken at the times indicated from the cannulated femoral arteries of the infused animals into heparinized tubes, and plasma β -glucuronidase activities were determined after centrifugation of the hematocrit tubes. ●: plasma levels in the enzyme-infused animals; ▲: plasma levels in the saline-infused control.

can be identified in rat liver and spleen 24 hr after infusion of human placental β -glucuronidase into the whole animal. The thermal inactivation of endogenous rat β -glucuronidase is illustrated by the results in the saline-infused rat. Assuming that the amount of enzyme which resists heating to 65° for 90 min in the enzyme-infused animal is human β -glucuronidase, 60% of the infused dose of human placental β -glucuronidase could be accounted for in liver, and 2.4% in spleen 24 hr after infusion. These results confirm the prediction from *in vitro* mixing experiments that infused human β -glucuronidase can be identified in homogenates of rat organs by its heat stability. Table 1 summarizes experiments showing inactivation of endogenous rat β -glucuronidase in Polytron homogenates of many rat organs by

heating homogenates to 65° for 90 min, and indicates the recoveries seen when varying amounts of human placental β -glucuronidase are added to the homogenates before heating. Although the recoveries shown in Table 1 indicate that human enzyme in rat organ homogenates may be slightly (about 10%) underestimated, no correction for this small difference was made in any of the data to be presented.

PLASMA DISAPPEARANCE AND LOCALIZATION OF INFUSED HUMAN PLACENTAL β -GLUCURONIDASE

Human placental β -glucuronidase is very poorly recognized and taken up by human fibroblasts in culture, suggesting that the majority of placental enzyme lacks the recognition factor necessary for specific recognition and uptake by human fibroblasts (5, 14). Cultured rat fibroblasts also discriminate between high uptake and low uptake forms of the human enzyme and take up very little human placental β -glucuronidase (9). Figure 2 shows the plasma disappearance curve of infused human placental β -glucuronidase in the rat. Plasma levels peak within 30 sec after infusion into the rat, and fall off rapidly with an initial half-disappearance rate of about 2.5 min. Over 90% of the infused enzyme is cleared from the rat plasma in 15 min. This experiment indicates that human placental β -glucuronidase, which is predominantly a low uptake enzyme in the human and rat fibroblast systems, is rapidly cleared from rat plasma and is not a low uptake enzyme *in vivo*.

When the same animals were killed 24 hr after infusion and organ homogenates were assayed for β -glucuronidase activity, the saline-infused control showed negligible amounts of heat-stable activity (Table 2). The enzyme-infused animal had significant amounts of heat-stable β -glucuronidase in liver and in spleen. In this experiment, 53.7% of the infused dose was recovered in these two organs 24 hr after infusion. No significant heat-stable β -glucuronidase was seen in homogenates of other organs. Thus, infused placental human β -glucuronidase appears to localize predominantly in liver and spleen.

PLASMA DISAPPEARANCE AND ORGAN LOCALIZATION OF INFUSED HUMAN PLACENTAL β -GLUCURONIDASE IN EVISCERATED RAT

The rapid clearance of infused human enzyme and its location predominantly in liver and spleen suggested that much of the infused dose might be cleared rapidly by the reticuloendothelial system. An experiment was carried out in an eviscerated animal to determine whether prevention of clearance by liver and spleen would increase the circulating life time of infused enzyme and allow uptake by other organs. Figure 3 compares the plasma

isappearance in the eviscerated animal to the plasma disappearance in the noneviscerated enzyme-infused control. Note that the plasma disappearance in the eviscerated animal is greatly prolonged ($t_{0.5}$ of 60 min) compared to that in the noneviscerated animal ($t_{0.5}$ of 3.25 min). Table 3 compares the organ distribution of heat-stable β -glucuronidase in these two animals. Soluble protein was determined for each organ homogenate in this experiment so that activity in each organ could be expressed as units per mg soluble protein as well as units per g tissue weight. Significant differences in soluble protein per g tissue homogenized were found only in the heart and bone homogenates. In the eviscerated rat, the liver, lung, kidney, heart, and spleen had significant amounts of heat-stable activity. The small amount of activity seen in liver indicates that circulatory isolation of the liver was not complete, presumably because its arterial circulation was not interrupted. Significant levels of

Table 2. Rat organ β -glucuronidase distribution 24 hr after infusion of human placental β -glucuronidase¹

	Control (saline infused)		Experimental	
	Total, units/g	Heat stable, units/g	Total, units/g	Heat stable, units/g
Liver	58,240	112 ²	69,440	19,520
Spleen	128,640	208 ²	151,040	12,864
Lung	14,384	64 ²	20,144	128 ²
Kidney	21,344	64 ²	18,464	64 ²
Brain	3,184	48 ²	3,424	48 ²
Heart	2,768	48 ²	2,432	48 ²
Skeletal muscle	1,520	0	1,424	32 ²

¹ Percentage of infused dose (250,000 units) recovered at 24 hr = 53.7%.

² Less than twice the nonenzymatic blank (256) for experiment.

Fig. 3. Disappearance of infused human β -glucuronidase from plasma in an eviscerated and a noneviscerated rat. The abdominal viscera including the spleen were removed from an anesthetized rat. The liver was left in place, but the portal vein was ligated. Each rat received an infusion of 500,000 units human placental β -glucuronidase in 1.0 ml buffered saline in the femoral vein. Blood samples were obtained at the times indicated from the cannulated contralateral femoral arteries as described in the legend to Figure 2, and plasma β -glucuronidase levels determined for the eviscerated (●) and the noneviscerated (▲) animal.

Table 3. Rat organ β -glucuronidase distribution 3 hr after infusion of human β -glucuronidase into eviscerated (Evisc.) and control rat

	Heat-stable β -glucuronidase, units/g		Soluble protein, mg/g		Heat-stable β -glucuronidase, units/mg soluble protein	
	Evisc.	Control	Evisc.	Control	Evisc.	Control
Liver	5,616	56,320	191	226.4	29.4	248.7
Lung	3,956	64 ¹	168	154	23.5	0.42
Kidney	3,120	48 ¹	178	160	17.5	0.30
Brain	208 ¹	64 ¹	115	121.6	1.8	0.53
Heart	1,647	101 ¹	196	137.6	8.4	0.73
Skeletal muscle	272 ¹	48 ¹	185	200.8	1.5	0.24
Skin	288 ¹		33		8.7	
Bone ²	17,242	438 ¹	120	71.7	143.7	6.11
Spleen ³		22,747		222.8		102.1

¹ Less than twice the nonenzymatic blank (256) for experiment.

² Bone activity was measured in homogenates of the rat femur. Each femur was isolated by dissection, frozen in liquid nitrogen, and pulverized by grinding with a mortar and pestle. The frozen powder was suspended in homogenization buffer and homogenized with the Polytron homogenizer as described for other organs under "Materials and Methods."

³ Spleen was removed from the eviscerated animal before infusion.

Fig. 4. Subcellular distribution of β -glucuronidase (74, 158 units/g) in liver from saline-infused rat. A 170-g rat received a 1.0-ml infusion of buffered saline (0.15 M NaCl, Tris-HCl, 0.01 M, pH 7.5). The liver was removed from the animal killed 24 hr later and fractionated as described in "Materials and Methods."

heat-stable β -glucuronidase in the noneviscerated animal were confined to liver and spleen, as found previously. The relatively large amount of heat-stable β -glucuronidase in the bone of the eviscerated animal suggests that reticuloendothelial elements in bone may capture significant amounts of infused enzyme if the liver and spleen are isolated or blocked.

SUBCELLULAR DISTRIBUTION OF INFUSED HUMAN PLACENTAL β -GLUCURONIDASE IN RAT LIVER

Subcellular fractions were prepared from homogenates of livers from rats killed 18 hr after infusion of saline or of purified human placental β -glucuronidase. The subcellular fractionation and the method of presenting the data are based on methods of De Duve *et al.* (7). The subcellular distribution profile of endogenous rat liver β -glucuronidase from a saline-infused rat is shown in Figure 4. The abscissa indicates the percentage of total

protein present in the subcellular fractions: nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and supernatant (S). The ordinate shows the relative specific activity of each subcellular fraction. The relative specific activity is calculated from the ratio of percentage of total enzyme activity in a particular subcellular fraction to the percentage of total protein in that fraction. By this method of presentation, a subcellular fraction enriched in an enzyme activity would show a relative specific activity greater than 1. The subcellular distribution pattern of β -glucuronidase shows the ML fraction to be highly enriched for β -glucuronidase activity. The previously reported enrichment of rat β -glucuronidase in the microsomal fraction of liver (7) was not seen in these experiments. Figure 5 presents the subcellular distribution profile of total, heat-stable, and heat-labile activity in homogenates of rat liver from a rat killed 18 hr after infusion of purified human placental β -glucuronidase. Of the total β -glucuronidase present in the original homogenate, 17% was heat stable. The overall pattern of subcellular distribution of heat-stable (human) β -glucuronidase is similar to that of total activity, and shows enrichment of the ML fraction. However, we have no explanation for the slight apparent enrichment of the nuclear fraction for heat-stable activity in this experiment. This was not observed in other similar experiments, all of which showed predominant enrichment of the heat-stable activity in the ML fraction. Heat-

Fig. 5. Subcellular distribution of total, heat-labile, and heat-stable liver β -glucuronidase activity in a β -glucuronidase-infused rat. A 170-g rat received 250,000 units human placental β -glucuronidase in 1.0 ml buffered saline. The liver was removed and fractionated 24 hr after the infusion. Total β -glucuronidase was measured for each fraction, as was heat-stable β -glucuronidase, that remaining after incubation of the subcellular fraction for 90 min at 65°. Heat-labile β -glucuronidase represents the difference between total and heat-stable β -glucuronidase activity in each subcellular fraction.

Fig. 6. Subcellular distribution of heat-labile and heat-stable β -glucuronidase activity in liver from a saline-infused rat with human β -glucuronidase added just before homogenization. To a 2 g-sample of rat liver from the animal described in the legend to Figure 4, 32,646 units human placental β -glucuronidase were added just before homogenization. Total β -glucuronidase activity and activity stable to heating for 90 min at 65° were determined for each subcellular fraction. Heat-labile β -glucuronidase activity represents the difference between total and heat-stable activities in each fraction.

labile activity, calculated by subtracting heat-stable activity in each fraction from the total activity in that fraction, shows the expected enrichment in the ML fraction.

Figure 6 shows the subcellular distribution of heat-labile and heat-stable β -glucuronidase in an experiment in which no β -glucuronidase was infused, but rather purified human placental β -glucuronidase was added to rat liver just before homogenization. It is clear that added β -glucuronidase does not show association with ML fraction, but, instead, is practically all associated with the microsomal and supernatant fractions. Thus, the evidence from these experiments indicates that infused human β -glucuronidase which is taken up by the liver has the expected distribution for localization in lysosomes.

TIME COURSE OF DISAPPEARANCE OF INFUSED ENZYME IN RAT LIVER AND RAT SPLEEN

The earlier experiments showed that infused human placental β -glucuronidase localizes preferentially in liver and spleen. To determine the half-life of enzyme which localizes in these organs, 12 170-g rats were infused with 250,000 units each of human β -glucuronidase and the amount of heat-stable β -glucuronidase remaining in these organs determined at various times after infusion. Figure 7 shows the results of this experiment. A linear regression analysis of these semilog plots of heat-stable β -glucuronidase activity yields half-disappearance times of 2.6 days for human enzyme in rat liver and 5.8 days for human enzyme in rat spleen.

PERIODATE TREATMENT OF HUMAN β -GLUCURONIDASE MODIFIES ITS CLEARANCE FROM PLASMA

Hickman *et al* (14) reported that the recognition component responsible for uptake of human lysosomal enzymes by fibroblasts was inactivated by periodate treatment. This finding provided tentative evidence that the recognition component was carbohydrate. Periodate treatment of human placental enzyme before infusion might also provide indirect evidence for carbohydrate involvement in the recognition system responsible for clearance of infused placental enzyme in the whole rat. Human placental β -glucuronidase was treated with periodate under conditions described in "Materials and Methods." Under these conditions, only slightly greater loss of catalytic activity (14%) occurred than in control enzyme (11%) which was exposed to

Fig. 7. Time course of disappearance of infused human placental β -glucuronidase in rat liver and spleen. Twelve 170-g rats received infusions of 250,000 units each of human placental β -glucuronidase in 1.0 ml buffered saline. Animals were killed at intervals over 9 days and the heat-stable β -glucuronidase activity measured in homogenates of liver and spleen. One animal died between 16 and 24 hr postinfusion and was not included in the analysis. Except for the 24-hr time point, two animals were killed at each time shown. Heat-stable β -glucuronidase activity is expressed as units per g organ weight.

Fig. 8. Effect of periodate treatment on plasma clearance of infused human placental β -glucuronidase. Human placental β -glucuronidase was treated with sodium metaperiodate as described in "Materials and Methods." Two 175-g rats were infused with 185,000 units of either periodate-treated (●) or non-periodate-treated (▲) human β -glucuronidase. Blood samples were withdrawn at times shown and plasma β -glucuronidase activities determined.

the same incubation conditions without added periodate, and the same subsequent dialysis. Although periodate treatment produced little loss of catalytic activity, the periodate treated enzyme differed from the non-periodate-treated enzyme in several ways. First, the periodate-treated enzyme had greatly reduced binding to concanavalin A (Con A)-Sephadex. Although the periodate-treated enzyme was quantitatively retained by Con A-Sepharose, less than 10% of the periodate-treated enzyme was bound to Con A-Sepharose and eluted with α -methylmannoside. Second, the periodate treated enzyme was considerably less stable to heating. The thermal inactivation conditions to which the control enzyme was stable (65° for 90 min) caused 77% inactivation of the periodate-treated enzyme. Third, the periodate-treated enzyme showed altered electrophoretic properties. Polyacrylamide gel electrophoresis of the control enzyme at pH 9.5 reveals only one sharp band of enzyme activity, whereas the periodate-treated enzyme shows one major band of activity (85%) with faster migration than control enzyme, and two smaller bands containing about 15% of the activity that migrate slower than control enzyme. No difference in apparent subunit size was detected between control and periodate-treated enzyme when examined in sodium dodecyl sulfate-containing polyacrylamide gels. Finally, gel filtration of the control enzyme on Ultrogel AcA-34 revealed a single symmetrical peak; the periodate-treated enzyme displayed some heterogeneity by this technique also. Although over 80% of the activity was found in the expected peak, nearly 20% eluted as a shoulder with apparent molecular weight between 1.5 and 2 times the apparent molecular weight of the control enzyme. This finding suggests that up to 20% of the activity may exist in an aggregated form in the periodate-treated enzyme.

Figure 8 compares the clearance from plasma of the infused control and periodate-treated human placental β -glucuronidase. It is evident that the periodate-treated enzyme has been converted into a long circulating form of the enzyme which is not cleared rapidly like the control enzyme. Since periodate treat-

ment destroys some of the carbohydrate structure of the human placental enzyme, as evidenced by the nearly complete abolition of binding to Con A-Sepharose, and also converts it into a slow clearance form, the recognition system responsible for rapid clearance of human placental enzyme from the rat may depend on recognition of carbohydrate residues on the enzyme.

DISCUSSION

Infused human placental β -glucuronidase is rapidly cleared from rat plasma and localizes predominantly in liver and spleen. Rapid clearance of infused enzyme from plasma of enzyme-deficient human subjects has also been observed for several human placental enzymes (3, 4, 6), for bovine liver β -glucuronidase in the mouse (8), and for several rat lysosomal enzymes infused into whole rats (22, 27). Slow clearance forms of lysosomal enzymes have also been reported (24, 26). Human placental β -glucuronidase, bovine liver β -glucuronidase, and rat β -glucuronidase (5, 23) are predominantly low uptake forms in the human fibroblast uptake system. These findings suggest a mechanism for rapid clearance of lysosomal enzymes *in vivo* that does not depend on the recognition component for uptake by fibroblasts. Periodate treatment largely destroys the clearance properties of human placental β -glucuronidase and rat preputial β -glucuronidase (27), suggesting that the clearance of these two enzymes depends on recognition of the carbohydrate structure of the enzyme. Two receptor-dependent clearance systems have been reported: one for clearance of glycoproteins with exposed galactose residues by hepatocytes (15, 19), and a second liver system which appears to recognize exposed *N*-acetylglucosamine residues (17, 18). Stahl and coworkers (28) concluded that the latter system is responsible for clearance of infused rat preputial gland β -glucuronidase, and several other rat lysosomal enzymes in the rat. Our studies, to be reported elsewhere, show that characterized glycoproteins also inhibit clearance of human placental β -glucuronidase from rat plasma, and support the suggestion based on the clearance properties of the periodate-treated enzyme, that the enzyme clearance depends on the carbohydrate structure of the enzyme.

The half-life determinations in these studies suggest shorter half-life of infused enzyme in rat liver and spleen than the greater than 2-week half-life previously found for platelet β -glucuronidase (24) and liver β -glucuronidase (16) taken up by human fibroblasts. It is possible that the half-life determinations in the studies reported here underestimate the true biologic half-life of infused enzyme since conversion to heat-labile enzyme may precede loss of biologic activity of the enzyme. The finding that periodate treatment of the enzyme reduces its heat stability provides an illustration of how this property of the enzyme can be lost without loss of catalytic activity. It should be pointed out that this reservation could also apply to our negative data on localization in other organs where rapid conversion of the infused enzyme to a heat-labile form (for which we have no evidence at present) would give the appearance of failure of heat-stable enzyme to localize in that organ.

The suggested preferential localization of infused enzyme in reticuloendothelial organs should be approachable directly with the model presented by examining the heat-stable β -glucuronidase in cell fractions after separation of rat parenchymal cells from Kupffer cells (20). If this localization were true for other lysosomal enzymes, as suggested by the findings of Stahl and coworkers for a number of rat lysosomal enzymes (22, 27), unmodified lysosomal enzymes from an organ source like placenta may be satisfactory for replacement in deficiency diseases such as Gaucher's disease, which involves the reticuloendothelial system. Human placental enzyme has been used in this disease by Brady and coworkers (3). Replacement in other lysosomal storage diseases which involve nonreticuloendothelial organs to an important degree may require choice of lysosomal enzymes with other localization properties, or finding methods to divert lysosomal enzymes from the liver-spleen recognition system to

other target tissues. Liposome packaging has been suggested as a means of targeting enzyme to other tissues and studied by several groups (8, 12, 30). The ready availability of human placental enzyme, its heat stability in rat tissues, and its relatively long biologic half-life *in vivo* makes the model presented here suitable for such studies. However, the special significance of the rat model presented is its suitability for determining whether human enzymes from different organ sources that have different recognition properties for human and rat fibroblasts will have different uptake, tissue distribution, or fate in the whole animal.

CONCLUSION

Human placental β -glucuronidase could be identified in rat organs after infusion by its stability to thermal inactivation conditions which destroy endogenous rat enzyme. Infused enzyme was rapidly cleared from rat plasma and localized predominantly in liver and spleen. Subcellular fractionation studies suggested that enzyme taken up by the liver was in lysosomes. Removal of abdominal viscera, including the spleen, and interruption of the portal circulation delayed the clearance of enzyme from plasma and resulted in substantial localization of enzyme in bone. Enzyme taken up by liver and spleen was relatively long lived with half-disappearance times of 2.6 and 5.8 days, respectively. Periodate treatment of the placental enzyme had little or no effect on catalytic activity, but dramatically altered its clearance properties converting it into a long circulating form of the enzyme.

REFERENCES AND NOTES

- Achord, D. T., Frankel, H., Glaser, J., Brot, F., and Sly, W. S.: Enzyme therapy: Rat model for β -glucuronidase replacement studies. *Amer. J. Hum. Genet.*, 27: 10A (1975).
- Brady, R. O., Pentchev, P. G., and Gal, A. E.: Investigations in enzyme replacement therapy in lipid storage diseases. *Fed. Proc.*, 34: 1310 (1975).
- Brady, R. O., Pentchev, P. G., Gal, A. E., Hibbert, S. R., and DeKaban, A. S.: Replacement therapy for inherited enzyme deficiency: Use of purified glucocerebrosidase in Gaucher's disease. *New Engl. J. Med.*, 291: 989 (1974).
- Brady, R. O., Tallman, J. F., Johnson, W. G., Gal, A. E., Leahy, W. R., Quirk, J. M., and DeKaban, A. S.: Replacement therapy for inherited enzyme deficiency: Use of purified ceramidetrihexosidase in Fabry's disease. *New Engl. J. Med.*, 289: 9 (1973).
- Brot, F. E., Glaser, J. H., Roozen, K. J., Sly, W. S., and Stahl, P. D.: *In vitro* correction of deficient human fibroblasts by β -glucuronidase from different human sources. *Biochem. Biophys. Res. Commun.*, 57: 1 (1974).
- De Barsey, T., Jacquemin, P., Van Hoof, F., and Hers, H. G.: Enzyme replacement in Pompe disease: An attempt with purified human α -glucosidase. In: R. J. Desnick, R. W. Bernlohr, and K. Krivit: *Enzyme Therapy in Genetic Diseases, Birth Defects: Original Article Series, Vol. 9*, pp. 184-190 (The Williams & Wilkins Co., Baltimore, 1973).
- DeDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F.: Tissue fractionation studies. VI. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.*, 60: 604 (1955).
- Desnick, R. J., Thorpe, S. R., and Fiddler, M. B.: Toward enzyme therapy for lysosomal storage diseases. *Physiol. Rev.*, 56: 57 (1976).
- Frankel, H. A., Glaser, J. H., and Sly, W. S.: Human β -glucuronidase. I. Recognition and uptake by animal fibroblasts suggests animal models for enzyme replacement studies. *Pediat. Res.*, 11: 811 (1977).
- Glaser, J. H., Roozen, K. J., Brot, F. E., and Sly, W. S.: Multiple isoelectric and recognition forms of human β -glucuronidase activity. *Arch. Biochem. Biophys.*, 166: 536 (1975).
- Glaser, J. H., and Sly, W. S.: β -Glucuronidase deficiency mucopolysaccharidosis: Methods for enzymatic diagnosis. *J. Lab. Clin. Med.*, 82: 969 (1973).
- Gregoriadis, G., and Ryman, B. E.: Fate of protein-containing liposomes injected into rats: An approach to the treatment of storage diseases. *Eur. J. Biochem.*, 24: 485 (1972).
- Hers, H. G.: Inborn lysosomal diseases. *Gastroenterology*, 48: 625 (1965).
- Hickman, S., Shapiro, L. J., and Neufeld, E. F.: A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. *Biochem. Biophys. Res. Commun.*, 57: 55 (1974).
- Kawasaki, T., and Ashwell, G.: Chemical and physical properties of an hepatic membrane protein that specifically binds asialoglycoproteins. *J. Biol. Chem.*, 251: 1296 (1976).
- Lagunoff, D., Nicol, D. M., and Pritzl, P.: Uptake of β -glucuronidase by deficient human fibroblasts. *Lab. Invest.*, 29: 449 (1973).
- Lunney, J., and Ashwell, G.: A hepatic receptor of avian origin capable of binding specifically modified glycoproteins. *Proc. Nat. Acad. Sci. U.S.A.*, 73: 341 (1976).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265 (1951).
- Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G.: The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Biol. Chem.*, 246: 1461 (1971).
- Munthe-Kas, A. C., Berg, T., and Seljelid, R.: Distribution of lysosomal enzymes in different types of rat liver cells. *Exp. Cell Res.*, 99: 146 (1976).
- Neufeld, E. F., and Cantz, M. J.: Corrective factors for inborn errors of mucopolysaccharide metabolism. *Ann. N. Y. Acad. Sci.*, 179: 580 (1971).
- Schlesinger, P., Rodman, J. S., Frey, M., Lang, S., and Stahl, P.: Clearance of lysosomal hydrolases following intravenous infusion. The role of liver in the clearance of β -glucuronidase and N-acetyl- β -D-glucosaminidase. *Arch. Biochem. Biophys.*, 177: 606 (1976).
- Sly, W. S., *et al.*: Unpublished observations.
- Sly, W. S., Glaser, J. H., Roozen, K., Brot, F. E., and Stahl, P.: Enzyme therapy in lysosomal storage diseases. In: J. M. Tager, G. J. M. Hooghwinkel, and W. T. Daems: *Enzyme Replacement Studies With β -Glucuronidase Deficiency*, pp. 288-291, (North-Holland Publishing Co., Amsterdam, 1974).
- Spiro, R. G.: Characterization of carbohydrate units of glycoproteins. *Methods/Enzymol.*, 8: 26 (1966).
- Stahl, P., Mandell, B., Rodman, J. S., Schlesinger, P., and Lang, S.: Different forms of rat β -glucuronidase with rapid and slow clearance following intravenous injection: Selective serum enhancement of slow clearance forms by organophosphate compounds. *Arch. Biochem. Biophys.*, 170: 536 (1975).
- Stahl, P., Rodman, J. S., and Schlesinger, P.: Clearance of lysosomal hydrolases following intravenous infusion. Kinetic and competition experiments with β -glucuronidase and N-acetyl- β -D-glucosaminidase. *Arch. Biochem. Biophys.*, 177: 594 (1976).
- Stahl, P., Schlesinger, P. H., Rodman, J., and Doebber, T.: Recognition of lysosomal glycosidases *in vivo* is inhibited by modified glycoproteins. *Nature*, 264: 86 (1976).
- Stockert, R. J., Morell, A. G., and Steinberg, H.: The existence of a second route for the transfer of certain glycoproteins from the circulation into the liver. *Biochem. Biophys. Res. Commun.*, 68: 988 (1976).
- Weissman, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlieb, A., and Nagle, D.: A general method for the introduction of enzymes by means of immunoglobulin-coated liposomes into lysosomes of deficient cells. *Proc. Nat. Acad. Sci. U.S.A.*, 72: 88 (1975).
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