Effects of Di-isopropyl Phosphorofluoridate on Rat Liver Microsomal and Lysosomal β -Glucuronidase

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(Received 16 November 1976)

iPr₂P-F (di-isopropyl phosphorofluoridate) administration to rats produces a liver-dependent specific elevation of plasma β -glucuronidase activity. The response is unaffected by puromycin pretreatment. By using subcellular-fractionation techniques, the rise in plasma β -glucuronidase activity was correlated temporally with a fall in liver microsomal β -glucuronidase activity. After iPr₂P-F treatment, liver microsomal membranes are depleted of β -glucuronidase but slowly return to normal over 1 week. On the other hand, liver lysosomal β -glucuronidase activity is high at early time points (less than 60 min) after iPr₂P-F administration but decreases to below control values; this lasts for a few days. The response to iPr₂P-F was demonstrated in isolated hepatocytes prepared from iPr₂P-F-treated rats. In such preparations, microsomal β -glucuronidase is lost rapidly, followed by a specific decrease in hepatocyte lysosomal β -glucuronidase. The results suggest that a pool of microsomal β -glucuronidase serves as precursor to plasma β -glucuronidase in iPr₂P-F-treated rats, and further, that microsomal β -glucuronidase may serve as precursor to lysosomal β -glucuronidase.

β -Glucuronidase (EC 3.2.1.31) is a unique lysosomal enzyme in that it is present in both endoplasmic reticulum and lysosomes of rodent liver (de Duve *et al.*, 1955). Previous studies from this laboratory have demonstrated that rat liver microsomal fractions and lysosomes contain distinct multiple forms of β -glucuronidase which can be resolved by isoelectric focusing (Owens *et al.*, 1975). Although the enzymes from these two liver organelles are catalytically identical, have the same molecular weight (Owens & Stahl, 1976) and, in the mouse, are the product of a single structural gene for β -glucuronidase (Swank & Paigen, 1973), the precise biosynthetic relationship between microsomal and lysosomal β -glucuronidase is unresolved.

Injection of iPr₂P-F (di-isopropyl phosphorofluoridate) (Stahl *et al.*, 1975) or other organophosphates (Williams, 1969; Suzuki *et al.*, 1975) into rats has been shown to elicit a massive increase in plasma β -glucuronidase within 60 min. The plasma activity of other lysosomal enzymes remains unchanged (Stahl *et al.*, 1975). The response is not cholinergically mediated (Stahl *et al.*, 1975), but is abolished by evisceration, suggesting that liver is the likely source of the plasma enzyme.

In the present study, it is demonstrated that the β -glucuronidase response to iPr₂P-F is independent of protein synthesis and that the source of plasma enzyme after iPr₂P-F injection is the liver microsomal pool of β -glucuronidase. Fractionation experiments demonstrate a temporal relationship between the fall in microsomal β -glucuronidase activity and the rise in plasma β -glucuronidase activity. Moreover, a similar relationship between the liver micro-

somal and lysosomal β -glucuronidase pools is suggested by the observation that a specific decrease in microsomal β -glucuronidase activity is temporally followed by a specific decrease in lysosomal β -glucuronidase.

Experimental

Materials

Female Wistar rats weighing approx. 200 g were obtained from National Laboratory Animals, St. Louis, MO, U.S.A. All reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., except [¹⁴C]leucine, which was obtained from New England Nuclear, Boston, MA, U.S.A. All solutions were prepared fresh on the day of use.

Enzyme assays

β -Glucuronidase was assayed by the method of Stahl & Touster (1971) with phenolphthalein monoglucuronide. *N*-Acetyl- β -D-glucosaminidase and β -galactosidase were assayed by using appropriate *p*-nitrophenyl glycosides as described by Stahl *et al.* (1976a). Glucose 6-phosphatase was measured as described by de Duve *et al.* (1955), with phosphate determined by the semidine method (Dryer *et al.*, 1957). Protein was measured by the Miller (1959) method, with bovine serum albumin as standard. One unit of enzyme activity is 1.0 μ mol of product formed/h under standard conditions.

Fractionation experiments

Rat liver was fractionated by a modification of the method of Owens *et al.* (1975). Animals were starved

overnight and killed by a blow to the head. Liver was homogenized in 3 vol. (3 ml/g) of 0.25 M-sucrose buffered with 0.050 M-imidazole, pH 7.2, in a Potter-Elvehjem homogenizer. All procedures were carried out at 4°C. Homogenates were fractionated into a nuclear fraction and a crude extract by low-speed centrifugation as described by de Duve *et al.* (1955). The crude extract, containing most of the cellular organelles, was then fractionated by centrifugation at 1.5×10^5 g-min. The resulting pellet or mitochondrial/lysosomal (ML) fraction was washed by resuspension in 2 vol. (2 ml/g) of 0.25 M-sucrose followed by recentrifugation. The supernatants from the two preceding spins were pooled and centrifuged at 4×10^6 g-min, yielding a pellet which corresponded to a microsomal fraction. The crude microsomal (P) fraction was resuspended in 4 vol. of 0.005 M-Tris/HCl, pH 7.5. Resuspension of crude microsomal fractions in hypo-osmotic buffer has been shown (Paigen, 1961; Owens *et al.*, 1975) to disrupt any lysosomes contaminating the microsomal preparations. The washed microsomal fractions after hypo-osmotic shock were collected by centrifugation at 3×10^6 g-min in a Spinco 35 rotor. The supernatants from the two preceding spins were pooled. Expression of data as relative specific activity is according to the suggestion of de Duve *et al.* (1955), where the specific activity of the isolated fraction is taken as a ratio to the specific activity of the starting homogenate. Triton X-100 (0.2%) was added to all fractions 45 min before assay.

Polyacrylamide-gel isoelectric focusing

Isoelectric focusing was performed as described by Owens *et al.* (1975). Gels were stained for β -glucuronidase activity by using naphthol-AS-BI-glucuronide and scanned at 500 nm in a Gilford gel scanner.

Isolation and fractionation of liver parenchymal cells

Liver parenchymal cells were isolated by a modification of the method of Seglen (1972). Rats were killed by a blow to the head. The liver was cannulated via the portal vein, after which the organ was transferred to a liver-perfusion chamber as described by Schlesinger *et al.* (1976). It was perfused (35 ml/min) for 6 min at 30°C, without recirculation, with Ca^{2+} -free buffer (8.3 g of NaCl, 0.5 g of KCl, 2.4 g of Hepes* per litre) adjusted to pH 7.6 with NaOH. Subsequently, the liver was perfused for 10 min at the same rate, with recirculation, by using a perfusate with Ca^{2+} (3.9 g of NaCl, 0.5 g of KCl, 0.7 g of CaCl_2 , 2.4 g of Hepes per litre) containing 0.1 mg of collagenase/ml (Sigma type 1). The perfused digested liver was compressed between two stainless-steel screens, dispersed in the collagenase-containing perfusate and

incubated at 37°C for 15 min with agitation. The cell preparation was filtered sequentially through gauze and 100 μm pore-size nylon mesh (Nytex Co., Tetko, Elmsford, NY, U.S.A.). The filtrate was centrifuged at 250 rev./min for 5 min in a Sorvall GLC-1 centrifuge. After centrifugation, the pellets were resuspended in 10 ml of Ca^{2+} -free Hanks balanced-salt solution and washed three times by centrifugation at 250 rev./min. The washed-cell preparation in Hanks solution was filtered through a 68 μm pore-size nylon mesh. The final cell preparation contained more than 90% parenchymal cells, as observed by light-microscopy, and viability was always greater than 80%, as assessed by the Trypan Blue-exclusion test. The glucose 6-phosphatase specific activity of isolated parenchymal-cell preparations was approx. 1.2 times that of the initial cell suspension. Glucose 6-phosphatase is found only in parenchymal cells, which comprise the bulk of the cells in liver.

Parenchymal cells were fractionated by a procedure that takes advantage of the osmotic stability of microsomal β -glucuronidase (Owens *et al.*, 1975). Freshly isolated cells were disrupted by suspension in cold hypo-osmotic buffer (0.005 M-Tris/HCl, pH 7.4) for 30 min, followed by homogenization in a loose-fitting Potter homogenizer (Kontes, Vineland, NJ, U.S.A.) (five strokes). The cell-free suspension was centrifuged for 10 min at low speed (1500 rev./min) in a Sorvall GLC-1 centrifuge. The resulting pellet was resuspended in 8 ml of 0.005 M-Tris/HCl, pH 7.4, homogenized with five strokes of a Potter homogenizer and centrifuged for 10 min at 1500 rev./min. The supernatants from the two initial spins were pooled and centrifuged at 3.33×10^6 g-min in a Spinco 50.1 rotor. The final supernatant contained most of the lysosomal enzymes, as measured by the appearance of β -galactosidase, a lysosomal marker enzyme. The pellet corresponds to a crude microsomal fraction containing most of the glucose 6-phosphatase and microsomal β -glucuronidase. The latter was determined by isoelectric-focusing experiments. The gels, stained specifically for β -glucuronidase (Owens *et al.*, 1975), revealed the exclusive presence of microsomal β -glucuronidase.

Results

Effect of inhibitors of protein biosynthesis on the response to $i\text{Pr}_2\text{P-F}$

The dependence of the plasma β -glucuronidase response to $i\text{Pr}_2\text{P-F}$ on protein biosynthesis was examined by using puromycin and cycloheximide as inhibitors. Puromycin (25 mg/kg) was injected intraperitoneally into animals 120 min before a standard dose (1 mg/kg) of $i\text{Pr}_2\text{P-F}$ and a tracer dose (10 μCi) of [^{14}C]leucine. Table 1 shows that although puromycin markedly inhibited incorporation of ^{14}C into trichloroacetic acid-precipitable protein, it had no

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Table 1. *Effect of puromycin on serum β -glucuronidase activity after iPr_2P-F administration*

Rats were injected intraperitoneally with 0.9% NaCl or puromycin (25 mg/kg) 120 min before a standard dose (1 mg/kg) of iPr_2P-F or 0.9% NaCl and a tracer dose (10 μ Ci) of [14 C]leucine. Animals were killed 60 min after the second injection; liver was homogenized in a Polytron homogenizer in 5 vol. of 0.005 M-Tris/sodium phosphate, pH 7.8. Portions of the homogenate were precipitated with a final concentration of 7.5% (w/v) trichloroacetic acid. The washed pellet was resuspended in Protosol (New England Nuclear) for counting of radioactivity.

Group	Pretreatment	Treatment	$10^{-4} \times [^{14}\text{C}]$ Leucine incorporation (c.p.m./g of liver)	Serum β -glucuronidase (units/ml)
1	0.9% NaCl	0.9% NaCl	26.7 ± 9.4	0.07 ± 0.01
2	0.9% NaCl	iPr_2P-F	37.6 ± 12	7.9 ± 1.2
3	Puromycin	0.9% NaCl	8.1 ± 2.6	0.07 ± 0.04
4	Puromycin	iPr_2P-F	7.5 ± 3.1	8.3 ± 0.9

Fig. 1. *Subcellular localization of β -glucuronidase and marker enzymes 90 min after iPr_2P-F treatment*

Rats were injected intraperitoneally with iPr_2P-F (1.25 mg/kg) or 0.9% NaCl 90 min before being killed. Livers were quickly removed and fractionated as described in the Experimental section. Fractions were assayed for β -glucuronidase (a), N -acetyl- β -D-glucosaminidase (b) and glucose 6-phosphatase (c). The upper graph in each case contains results from iPr_2P-F -treated rats, and the lower graph contains those of controls. Data were taken from three separate experiments with two rats/group. Recoveries of β -glucuronidase activity were control, 95%, experimental, 90%; of N -acetyl- β -D-glucosaminidase, control, 87%; experimental, 88%; of glucose 6-phosphatase, control, 90%, experimental, 93%. N, Nuclear fraction; ML, mitochondrial/lysosomal fraction; P, microsomal fraction; S, supernatant fraction. Relative specific activity is the specific activity of the isolated fraction/specific activity of homogenate, as described by de Duve *et al.* (1955). The width of each bar graph indicates the percentage of the total protein recovered in the fraction. Vertical lines indicate S.E.M.

effect on the plasma β -glucuronidase response. In control experiments, puromycin alone had no effect on plasma β -glucuronidase and iPr_2P-F alone had no effect on [14 C]leucine incorporation into protein. Similar experiments with cycloheximide yielded similar results, except that cycloheximide alone occasionally increased plasma β -glucuronidase severalfold. The response was quite variable and was sometimes absent, making quantification difficult. The cycloheximide-induced increase in plasma β -glucuronidase remains unexplained.

Effect of iPr_2P-F on the subcellular distribution of liver β -glucuronidase

Experiments of Stahl *et al.* (1975) suggested that liver was the primary source of plasma β -glucuronidase after organophosphate poisoning. It was considered reasonable therefore to test liver subcellular fractions for β -glucuronidase activity after iPr_2P-F administration, since (i) β -glucuronidase has a unique subcellular distribution, with a considerable portion of its activity associated with endoplasmic reticulum

Table 2. *Effect of iPr₂P-F on the subcellular distribution of rat liver β -glucuronidase*

Rats were injected with iPr₂P-F as described in Fig. 1. (a) The activity occurring in the nuclear (N), mitochondrial/lysosomal (ML), microsomal (P) and supernatant (S) fractions are expressed as a percentage of the total activity occurring in the homogenate. Activity/g of liver refers to units of β -glucuronidase or mg of protein. Six animals were used in each experiment. Recovery is (activity in isolated fractions/activity in homogenate) $\times 100$. (b) Specific activity (units/mg of protein) of β -glucuronidase in fractions described in (a) in control ($-iPr_2P-F$) and experimental ($+iPr_2P-F$) rats.

	Presence of iPr ₂ P-F	Activity/g of liver	Percentage of total				Recovery (%)
			N	ML	P	S	
(a) Percentage values							
β-Glucuronidase	—	79	21	31	27	17	96
	+	72	17	45	5	25	92
Protein	—	185	33	14	8	33	88
	+	188	40	12	7	26	85
(b) Specific activity							
β-Glucuronidase	—		0.27	0.95	1.44	0.22	—
	+		0.16	1.43	0.27	0.37	—

Fig. 2. Time course of the iPr₂P-F effect on rat liver microsomal β -glucuronidase and rat serum β -glucuronidase

Rats were injected intraperitoneally with iPr₂P-F (1.25 mg/kg) or 0.9% NaCl, and killed at appropriate times. Serum samples were taken and livers fractionated as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of results from three to four rats. ○, Ratio of microsomal β -glucuronidase specific activity of iPr₂P-F-treated rats (A) to the specific activity of control rats (B); ●, serum β -glucuronidase (units/ml).

a differential-centrifugation method (Owens *et al.*, 1975) which uses hypo-osmotic shock of the isolated microsomal fraction. This lowers lysosomal contamination. The results (Fig. 1, Table 2) demonstrate that microsomal fractions from iPr₂P-F-treated rats were depleted of their β -glucuronidase, whereas marker enzymes *N*-acetyl- β -D-glucosaminidase, β -galactosidase (not shown) and glucose 6-phosphatase displayed normal distributions. Absolute values for the distribution of β -glucuronidase and protein (Table 2) indicate that the decrease in the specific activity of microsomal β -glucuronidase was due to loss of enzyme activity from the fraction, since protein content was unaffected by iPr₂P-F treatment. There was a slight increase in β -glucuronidase activity associated with the lysosomal fraction (see Fig. 4), which may reflect uptake of the enzyme by the liver (Stahl *et al.*, 1975).

Correlation of loss of liver microsomal β -glucuronidase activity with the increase in the plasma enzyme

The evidence suggests that microsomal β -glucuronidase (or a certain pool of microsomal β -glucuronidase) serves as a precursor to plasma β -glucuronidase after iPr₂P-F treatment. To explore the temporal relationship between microsomal and plasma β -glucuronidase after iPr₂P-F treatment, experiments were undertaken at selected times after iPr₂P-F administration, in which plasma β -glucuronidase and liver microsomal β -glucuronidase were measured in the same animal. Microsomal fractions were isolated as described above by using a hypo-osmotic wash to decrease lysosomal contamination. Fig. 2 demonstrates a correlation between the fall in liver microsomal β -glucuronidase activity (units/mg of protein) after iPr₂P-F treatment and the rise in plasma β -glucuronidase activity (units/ml of plasma).

(Owens *et al.*, 1975) and (ii) the plasma response to iPr₂P-F is limited to β -glucuronidase and excludes other lysosomal enzymes (Stahl *et al.*, 1975). Fractionation experiments were undertaken 90 min after administration of iPr₂P-F. Subcellular fractions were prepared from control and experimental animals by

Fig. 3. Polyacrylamide-gel isoelectric focusing of β -glucuronidase extracted from microsomal fractions of control and iPr_2P -F-treated rats

Rats were injected intraperitoneally with iPr_2P -F (1 mg/kg) and killed 90 min later. Microsomal fractions, prepared as described in the Experimental section, were extracted into 0.2% Triton X-100, buffered with 0.005 M-Tris/HCl, pH 7.4, followed by high-speed centrifugation. Gels were run as described by Owens *et al.* (1975) by using pH 5–7 Ampholite in 6 M-urea, and stained for β -glucuronidase activity. A Gilford gel scanner was used at 500 nm to quantify the staining intensity of the gels. Reference purified liver microsomal β -glucuronidase and β -glucuronidase from serum of iPr_2P -F-treated rats was prepared by antibody–Sephadex chromatography as described by Stahl *et al.* (1975) and Owens & Stahl (1976). Reference enzyme (0.2 unit) was applied to the gels: (a) 0.2 unit of purified microsomal β -glucuronidase [M_1 , M_2 , M_3 refer to the three isoelectric forms which normally occur in rat liver microsomal fractions (Owens *et al.*, 1975)]; (b) 1 mg of Triton X-100-solubilized microsomal protein from control rat; (c) 1 mg of Triton X-100-solubilized microsomal protein from iPr_2P -F-treated rats; (d) 0.2 unit of β -glucuronidase isolated from serum of iPr_2P -F-treated rats by antibody–Sephadex chromatography.

Liver glucose 6-phosphatase, *N*-acetyl- β -D-glucosaminidase and β -galactosidase were unaffected. The specific depletion of liver microsomal β -glucuronidase was tested by polyacrylamide-gel isoelectric-focusing experiments on enzyme isolated from microsomal fractions of control and iPr_2P -F-treated rats. The gels were stained specifically for β -glucuronidase, and scans of stained gels appear in Fig. 3. Microsomal β -glucuronidase has been shown by Owens *et al.* (1975) to be composed of three closely associated forms (M_1 , M_2 , M_3), which can be resolved by polyacrylamide-gel isoelectric focusing. Fig. 3 indicates that all three forms of microsomal β -glucuronidase are depleted after iPr_2P -F treatment. Moreover, there appears to be more intense staining of β -glucuronidase activity in the acid region of the gel when enzyme from experimental microsomal

fractions was used. Since β -glucuronidase isolated from plasma of iPr_2P -F-treated rats has a very acid isoelectric point (Fig. 3d), the increase in microsomal acidic forms could be due to contamination of the microsomal fraction by β -glucuronidase-rich plasma. To rule out this possibility, excess β -glucuronidase (i.e. β -glucuronidase isolated by immunoaffinity chromatography from plasma of iPr_2P -F-treated rats; Stahl *et al.*, 1975) was added to a sample of liver homogenate before fractionation. The added enzyme was accounted for in the supernatant by a comparison of β -glucuronidase activity in the various fractions with that in identical fractions from a control fractionation (no β -glucuronidase added). Incubation of purified microsomal β -glucuronidase with rat plasma did not result in any alteration of the enzyme, as monitored by isoelectric focusing.

Long-term effects on iPr₂P-F on liver microsomal and lysosomal β -glucuronidase

Since microsomal β -glucuronidase is depleted after iPr₂P-F treatment, it was decided to follow microsomal and lysosomal β -glucuronidase activity over several days. Animals were injected intraperitoneally with either 0.9% NaCl or iPr₂P-F, and killed at selected times. Lysosomal and microsomal fractions were prepared as described above. Within minutes after iPr₂P-F treatment (Fig. 4), the microsomal β -glucuronidase activity fell precipitously, returning to control values by about 7 days. On the other hand, the lysosomal β -glucuronidase activity was slightly elevated in the first few hours after iPr₂P-F treatment but fell to much lower values over the 7-day experiment. Control marker-enzyme activities were unchanged in control and experimental animals.

Long-term effects of iPr₂P-F on microsomal and lysosomal β -glucuronidase from isolated liver parenchymal cells

β -Glucuronidases, in general, have been shown to have rather short plasma survival times after intravenous injection. Purified liver lysosomal and microsomal β -glucuronidases are cleared very rapidly ($t_{1/2} < 5$ min) after intravenous injection. On the

Fig. 4. Long-term effects of iPr₂P-F on the subcellular distribution of β -glucuronidase

Rats were injected intraperitoneally with iPr₂P-F (1.25 mg/kg) or 0.9% NaCl and killed at selected times. Liver was fractionated as described in the Experimental section. Fractions were treated with 0.2% Triton X-100 before assay. Data for β -glucuronidase are expressed as a percentage of the corresponding 0.9% NaCl-injected control which was fractionated simultaneously. The results are means $\pm$ S.E.M. of three experiments, with two rats per experiment. ●, β -Glucuronidase activity of mitochondrial/lysosomal fraction; ○, β -glucuronidase activity of washed microsomal fraction.

Fig. 5. Effect of iPr₂P-F on lysosomal and microsomal glucuronidase of rat liver parenchymal cells

Rats were injected with iPr₂P-F (1.25 mg/kg) and killed at selected times. Livers were perfused with collagenase, and parenchymal cells were isolated as described in the Experimental section. Isolated cells were fractionated into osmotically solubilized (lysosomal; ●) β -glucuronidase and detergent-solubilized (microsomal; ○) β -glucuronidase as described in the Experimental section. Data are expressed as percentage of the corresponding 0.9% NaCl-injected control.

contrary, β -glucuronidase isolated from plasma of iPr₂P-F-treated rats, is cleared slowly ($t_{1/2} > 30$ min) after intravenous injection. Irrespective of the rate of clearance, all three enzymes are cleared by the liver (Stahl *et al.*, 1975). Recent work from this laboratory (P. Stahl & M. J. Miller, unpublished work) has indicated that uptake of lysosomal enzymes by liver is mediated by liver Kupffer cells. Consequently, uptake of plasma β -glucuronidase by liver Kupffer cells after iPr₂P-F treatment would tend to cloud any specific changes which might have occurred in liver parenchymal-cell lysosomes. To rule out any contribution made by Kupffer cells, liver parenchymal cells were isolated by the collagenase-perfusion technique

before fractionation. Details of the cell-isolation procedure are given in the Experimental section. Parenchymal cells were prepared from livers of control and iPr_2P -F-treated rats at selected times after treatment. The isolated cells were disrupted and fractionated by a method that allows separation of microsomal (i.e. osmotically insensitive) and lysosomal (i.e. osmotically sensitive) β -glucuronidase. The results (Fig. 5) indicate that whereas microsomal β -glucuronidase activity fell sharply, exactly as was found in whole liver, the pattern for lysosomal β -glucuronidase was different in parenchymal cells from that in whole liver. The increase in lysosomal β -glucuronidase activity seen in whole liver during the first few hours after drug treatment was not seen in isolated parenchymal cells, but the activity of lysosomal β -glucuronidase fell slowly over the 4-day experiment.

Discussion

Organophosphate compounds elicit a massive selective increase in plasma β -glucuronidase activity. Stahl *et al.* (1975) have implicated the liver as the source of the plasma enzyme after iPr_2P -F treatment, since evisceration abolished the iPr_2P -F effect. Moreover, studies with Diazinon, an organophosphate insecticide, have demonstrated specific effects on liver microsomal β -glucuronidase (Suzuki *et al.*, 1975).

The results of the present study corroborate the findings of Suzuki *et al.* (1975) and demonstrate that a pool of liver microsomal β -glucuronidase serves as a precursor to plasma β -glucuronidase after organophosphate stimulation. The results do not exclude the presence of an intermediate pool of β -glucuronidase of unknown subcellular localization. The latter could be involved in this response, since, strictly speaking, a precursor-product relationship requires that a specific population of prelabelled molecules is first identified as precursor and subsequently as product. This is not possible at present because of the low biosynthetic rate of β -glucuronidase (Van Lanker & Lentz, 1970; Wang & Touster, 1975; J. W. Owens & P. Stahl, unpublished work) and other lysosomal enzymes (Warburton & Wynn, 1976). Liver-fractionation experiments revealed a dramatic fall in microsomal β -glucuronidase activity. Other marker enzymes, glucose 6-phosphatase and *N*-acetyl- β -D-glucosaminidase, as well as protein content of the fractions, remained unchanged. Moreover, a temporal relationship was established between the decrease in microsomal β -glucuronidase activity and the increase in plasma β -glucuronidase activity. Isoelectric-focusing experiments confirmed the loss of β -glucuronidase from microsomal membranes.

Liver microsomal β -glucuronidase, in contradistinction to its lysosomal counterpart, is a membrane-

bound hydrolase which can be solubilized with detergents but not by osmotic shock (Owens *et al.*, 1975). The function of the microsomal β -glucuronidase is unclear. An attractive possibility, suggested by various studies (Van Lanker & Lentz, 1970; Owens *et al.*, 1975), is that rat microsomal β -glucuronidase serves as a precursor to liver lysosomal β -glucuronidase, with which it is catalytically and immunologically very similar, if not identical (Owens & Stahl, 1976). Alternatively, microsomal β -glucuronidase may be a secretory form of the enzyme, only entering lysosomes by the circuitous route of pinocytotic uptake after secretion. Previous results have shown that β -glucuronidase, isolated from plasma of iPr_2P -F-treated rats, is cleared by liver (Stahl *et al.*, 1975) when re-injected into the anaesthetized rat.

The long-term experiments reported in the present study revealed a short-lived increase in liver lysosomal β -glucuronidase in the first few hours after iPr_2P -F administration. Subsequently, lysosomal β -glucuronidase activity fell to about 70% of control values over the 7-day experiment. Microsomal β -glucuronidase activity, on the other hand, fell precipitously in the first few hours after iPr_2P -F injection and slowly returned to normal over 1 week. The rate of return of microsomal β -glucuronidase to pre-injection activity is consistent with turnover studies, showing that microsomal β -glucuronidase has a $t_{1/2}$ of about 4 days (Owens, 1976). The short-lived peak in lysosomal β -glucuronidase after iPr_2P -F treatment may represent Kupffer-cell lysosomal uptake by pinocytosis during the period immediately after drug administration when plasma β -glucuronidase activity is highest. Stahl *et al.* (1975) have demonstrated that although clearance *in vivo* of β -glucuronidase isolated from the serum of iPr_2P -F-treated rats is sluggish when compared with other forms of β -glucuronidase, the clearance that does occur is exclusively due to liver. Work from this laboratory (P. Stahl & M. J. Miller, unpublished work) and others (Achord *et al.*, 1977) indicates that uptake of lysosomal enzymes by liver is mediated by Kupffer cells. For this reason, a long-term study was undertaken with isolated liver parenchymal cells to determine the effects of iPr_2P -F on the subcellular distribution of β -glucuronidase. β -Glucuronidases from the two subcellular sites were separated on the basis of their sensitivity to osmotic (lysosomal) and detergent (microsomal) extraction (Owens *et al.*, 1975). The results indicate that, as expected, the specific activity of microsomal β -glucuronidase was rapidly decreased after iPr_2P -F administration. However, the early rise in lysosomal β -glucuronidase activity was not seen. Lysosomal β -glucuronidase activity slowly decreased over the period (4 days) of the experiment. Of particular note is the observation that the decrease in lysosomal β -glucuronidase activity temporally followed the decrease in microsomal β -glucuronidase activity.

The working hypothesis, partially supported by this study, is that microsomal β -glucuronidase, a liver parenchymal-cell enzyme, serves as a precursor to both plasma β -glucuronidase and/or liver parenchymal-cell lysosomal β -glucuronidase. The decrease in microsomal β -glucuronidase caused by iPr_2P -F treatment results in a delayed, but sizeable, fall in lysosomal β -glucuronidase activity. Kupffer-cell uptake of β -glucuronidase in the first few hours after iPr_2P -F treatment masks the changes occurring in parenchymal-cell lysosomes when whole liver is fractionated. These conclusions are tentative and must be supported by more precise fractionation experiments on both isolated Kupffer and parenchymal cells.

With respect to the secretion of microsomal β -glucuronidase by liver, highly purified rat liver microsomal β -glucuronidase has an isoelectric point of 6.7 and is rapidly cleared after intravenous injection. Both β -glucuronidase isolated from plasma of iPr_2P -F-treated rats and β -glucuronidase purified from normal rat serum have isoelectric points of 5.4 and are slowly cleared *in vivo* (Stahl *et al.*, 1975). As shown here, incubation of microsomal enzyme with plasma does not result in any change in the pI. The earlier observations, together with others reported in the present paper, suggest that microsomal β -glucuronidase must be modified before secretion by a post-translational mechanism. Lysosomal enzymes have been shown to have recognition sites associated with them which mediate their uptake into cells (Hickman & Neufeld, 1972; Stahl *et al.*, 1976a,b,c). It is likely that the recognition site that is associated with purified microsomal β -glucuronidase is masked or deleted from microsomal β -glucuronidase before enzyme secretion. The concept that cells secrete enzyme that is devoid of recognition site(s) has been advanced by observations from this laboratory (Stahl *et al.*, 1976c), where certain forms of β -glucuronidase (e.g. normal rat serum β -glucuronidase) have been found to have long plasma survival times. Hickman & Neufeld (1972), Glaser *et al.* (1974) and Vladutiu & Rattazzi (1975) have demonstrated that culture media supporting cells from patients with I-cell disease (mucopolipidosis type II) contain lysosomal enzymes that are not recognized or taken up by fibroblasts. A physiological role for lysosomal enzymes in extracellular fluids remains to be established.

The help of Ms. Jane S. Rodman is gratefully acknowledged. We also acknowledge Dr. Paul Schlesinger and Dr. W. S. Sly for many useful discussions and comments. This work was supported by U.S. Public Health Service grants CA 12858 and GM21096.

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