Intracellular Transport of Mouse Kidney β -Glucuronidase Induced by Gonadotrophin

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1. The response of renal β -glucuronidase with time to the injection of gonadotrophin was investigated in each submicrosomal fraction of rough and smooth microsomal fractions of mouse kidney homogenate. 2. The increase in β -glucuronidase activity appeared initially in membranes of the rough microsomal fraction, 24 h after injection. 3. Afterwards the newly synthesized enzyme appeared in the contents of the rough microsomal fraction and was subsequently found in the smooth microsomal fraction, reaching a maximum concentration in this fraction at 72 h. 4. At this juncture, a decrease in the enzyme activity was observed in rough microsomal contents whereas the lysosomal fraction had reached its maximum value. 5. The time-course of the appearance of β -glucuronidase in the submicrosomal fractions after the gonadotrophin stimulation suggests that the newly synthesized enzyme at the site of membrane-bound ribosomes is transferred across the membrane into cisternae of the rough endoplasmic reticulum, and then is transported into lysosomes via the smooth endoplasmic reticulum. 6. The properties of microsomal and lysosomal β -glucuronidases were compared.

The intracellular mechanism responsible for the delivery of acid hydrolases from the endoplasmic reticulum to the lysosomes is poorly understood. Kato *et al.* (1970) have described an experiment with mouse kidney in which the separate incorporation of [^{14}C]glucosamine and [^{14}C]leucine into microsomal and lysosomal β -glucuronidase was measured 3 h after the injection of each tracer. Approx. 80% of the newly synthesized enzyme appeared in the microsomal fraction and approx. 20% was transferred to the lysosomes at 72 h after stimulation by gonadotrophin. These results suggested that the mouse renal β -glucuronidase is a glycoprotein and that the newly synthesized enzyme is transferred from the endoplasmic reticulum to the lysosomes. This suggestion was also supported by the progressive increase in specific activity of microsomal β -glucuronidase observed during the first 36 h of the stimulation before an increase in specific activity of the lysosomal enzyme was detected. Therefore the response of mouse renal β -glucuronidase to gonadotrophin may permit one to trace the route of intracellular transport of a lysosomal enzyme.

In the present paper, we describe a study of the time-course of the appearance of β -glucuronidase in submicrosomal fractions of rough and smooth microsomal fractions after stimulation by gonadotrophin. Further, the intramicrosomal status of this enzyme protein is discussed, and the properties of

microsomal and lysosomal β -glucuronidases have also been compared.

Experimental

Animals

Male DDD mice (Institute of Medical Science, University of Tokyo, Tokyo, Japan), 10–15 weeks old and weighing 28 ± 3 g, were used. The animals were starved for 15 h before death. All injections were made intraperitoneally.

Methods

Tissue fractionation. The animals were killed by cervical dislocation. Their kidneys were rapidly excised and chilled in ice-cold 0.25 M-sucrose–1 mM-EDTA. The chopped kidney pieces were mixed with 3 vol. of the sucrose–EDTA solution and homogenized by five strokes with a Teflon homogenizer (radial clearance of 0.15 mm) of the Potter–Elvehjem type (1500 rev./min). Cell debris and the nuclei were removed by centrifugation at $650g_{av}$ for 2 min. The supernatant was successively centrifuged at $10000g_{av}$ for 20 min and then at $105000g_{av}$ for 60 min to isolate the mitochondria–lysosome fraction and the microsomal fraction respectively. Both fractions were usually washed once by resuspending

them in sucrose-EDTA solution and by spinning down again.

When studying the response of β -glucuronidase intracellular fractions to gonadotrophin, homogenization was performed by a slight modification of the method described by de Duve *et al.* (1955). The tissue was cut into a few pieces and dispersed with approx. 3 vol. of the sucrose-EDTA solution in the same Teflon homogenizer as used above. The tube, kept in ice, was given a single run upward against the rotating pestle (1500 rev./min). The sediment was re-homogenized in the same quantity of the medium and centrifuged at 150 g_{max} for 2 min. After this operation had been repeated a second time, the supernatants were combined and made up to volume, to form the 1:10 (g of original wet tissue/ml) cytoplasmic extract. The combined supernatant was fractionated into three particulate fractions and a soluble fraction by centrifugation successively at 650 g_{av} for 2 min (nuclei and cell debris), at 10000 g_{av} for 5 min (mitochondria-lysosomes), and at 105000 g_{av} for 60 min (microsomal fraction). The remaining supernatant represented the soluble fraction. The three particulate fractions were resuspended in sucrose-EDTA solution.

Analytical procedures. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. Phospholipids and RNA were determined by the methods described by Ito & Sato (1969), except that RNA extraction was done at 70°C for 20 min and not at 90°C for 15 min. Phosphate was determined by the method of Bartlett (1959).

Enzyme assays. β -Glucuronidase activity was determined by the method of Kato *et al.* (1960) with *p*-nitrophenyl glucuronide as substrate. Acid phosphatase was assayed with *p*-nitrophenyl phosphate as substrate (Shibko & Tappel, 1965). Glucose 6-phosphatase was determined by the method of Swanson (1955). Specific activities are expressed as nmol of substrate hydrolysed/min per mg of protein.

Heat-inactivation of β -glucuronidase. Enzyme preparations were adjusted to pH 4.5 (sodium acetate buffer), pH 6.7 (sodium phosphate buffer) and pH 7.0 (sodium phosphate or tris-HCl buffer), respectively (final buffer concentration, 0.02 M). Samples (0.5 ml) of each buffered enzyme solution were incubated in separate tubes at 70°C for periods of up to 60 min; then they were chilled and adjusted to pH 4.5 by the addition of 0.4 ml of 0.4 M-sodium acetate buffer, pH 4.5. Finally, the remaining enzyme activity was measured. The pH of each sample remained unchanged before and after the incubation at 70°C.

Separation of rough and smooth microsomal sub-fractions. Subfractionation of microsomal fraction was performed by a slight modification of the method described by Dallner (1963). The 10000 g_{av} super-

natant obtained from the 25% (w/v) kidney homogenate was mixed with 1 M-CsCl to give a final CsCl concentration of 15 mM. The 6 ml of the Cs⁺-containing 10000 g_{av} supernatant was layered carefully over 4 ml of 1.3 M-sucrose-15 mM-CsCl, and centrifuged in a no. 50 Spinco rotor at 150900 g_{av} for 120 min. During centrifugation a double fluffy layer formed at the interface (smooth microsomal fraction) and a brown pellet formed at the bottom (rough microsomal fraction). The fluffy layers were separated, then pooled and diluted with water to a sucrose concentration of 0.25 M. This mixture was then centrifuged at 105000 g_{av} for 90 min. The resulting pellet was rinsed three times with 0.25 M-sucrose and resuspended in the same solution to give the smooth microsomal fraction. The rough microsomal fraction at the bottom was recovered by removing the remainder of the 1.3 M-sucrose. The rough microsomal fraction was resuspended in 0.25 M-sucrose and centrifuged at 105000 g_{av} for 90 min. The pellet was rinsed three times with 0.25 M-sucrose and resuspended in the same solution to give a suspension of rough microsomal fraction.

Separation of submicrosomal fractions by deoxycholate treatment. Microsomal membrane components and contents were separated by the method of Dallner *et al.* (1966). Both rough and smooth microsomal fractions were treated with 0.26% sodium deoxycholate (final concentration) and were centrifuged for 4 h at 105000 g_{av} to yield tightly packed pellets consisting of ribosomes and membranes, and supernatants which represent the microsomal contents. The pellets were rinsed three times with 0.25 M-sucrose, then were resuspended in the same solution and treated with 0.5% sodium deoxycholate (final concentration). The clarified suspensions were centrifuged at 105000 g_{av} for 2 h to yield ribosome pellets and supernatants containing the membrane components in soluble form. The supernatants thus prepared from rough and smooth microsomal fractions will be referred to as 'rough microsomal contents', 'rough microsomal membranes', 'smooth microsomal contents' and 'smooth microsomal membranes', respectively.

Variation with time of subcellular and submicrosomal β -glucuronidase response to gonadotrophin. The mice were arranged in nine groups of five animals. The mice of eight groups were injected intraperitoneally with 2.5 i.u. of human chorionic gonadotrophin (Teikokuzoki Seiyaku Co., Tokyo, Japan) in 0.9% NaCl and killed at appropriate intervals up to 168 h after the injection; one group was injected with 0.9% NaCl and served as a control for the other groups.

To evaluate various fractions of microsomal material, a larger number of animals was required. Thus, the mice were arranged in six groups of 25 animals: five groups received gonadotrophin as

described above, and one group received 0.9% NaCl. The separation of rough and smooth microsomal fractions and microsomal membranes and the contents of each microsomal subfraction were performed by the methods described above. The β -glucuronidase activity of each subcellular and submicrosomal fraction was measured and the percentage increase in activity relative to the control was calculated.

Ultrasonic disintegration of microsomal fraction. Ice-cold samples (4ml) of microsomal suspension (5mg of protein/ml) in a tube (18mm diam.) was sonicated for different times (1, 2, 3 and 5 min) with a Sonore 150 S ultrasonic disintegrator (Umeda Electronic Co., Tokyo, Japan) operated at 20 000 Hz. The clarified suspension was then centrifuged at 105 000 g_{av} for 60 min. The tightly packed pellet was resuspended in 0.25M-sucrose to give a microsomal-membrane fraction consisting of ribosomes and membranes. The supernatant represented microsomal contents together with the portion of the membrane components that are no longer sedimentable.

Tryptic digestion of microsomal fraction. Trypsin treatment of kidney microsomal fraction was done by the method of Omura *et al.* (1967). Washed microsomal fraction was suspended in 0.1M-sodium phosphate buffer, pH 7.5 (20mg of protein/ml), and 3mg of crystalline trypsin, dissolved in 1ml of 2M-HCl, was added to each 10ml of the microsomal suspension. The suspension was incubated overnight at 0°C. After the addition of an aqueous solution of the same amount of soya-bean trypsin inhibitor, the suspension was centrifuged at 105 000 g_{av} for 60 min. The pellet was washed once with 0.25M-sucrose and was resuspended in the same medium to give trypsin-digested microsomal fraction.

Polyacrylamide-gel electrophoresis of β -glucuronidase. To obtain solubilized β -glucuronidases of microsomal contents and membranes, submicrosomal fractions were prepared by the deoxycholate treatment described above. The membrane fraction in 0.5% deoxycholate solution was left overnight at 0°C to solubilize β -glucuronidase from the membranes. Each submicrosomal fraction in deoxycholate solution was adjusted to pH 4.5 with 0.4M-sodium acetate buffer (pH 4.5), and the deoxycholic acid was extracted with 0.33 vol. of ether. The ether extracts were pipetted off and the same extraction was repeated three times. Insoluble materials that appeared in aqueous layers after the extraction were removed by low-speed centrifugation and were discarded. The supernatant solutions contained the solubilized β -glucuronidases from submicrosomal fractions. The solubilized lysosomal β -glucuronidase was obtained after the mitochondria-lysosome fraction had been frozen and thawed 10 times and then centrifuged at 105 000 g_{av} for 60 min. Each super-

natant solution thus obtained was subjected to polyacrylamide-gel electrophoresis by the method of Davis (1964) (in tris-glycine buffer, pH 8.6). The gels were stained for β -glucuronidase by the post-coupling azo-dye method of Fishman & Goldman (1965), which results in an intense non-diffusing red band in regions of glucuronidase activity. The gels were incubated in 0.4M-sodium acetate buffer, pH 4.5, containing 40 μ M-naphthol AS-BI glucuronide for 5–20 min at 37°C. After incubation the gels were washed with water and then dipped in diazotized pararosaniline solution (Hayashi *et al.*, 1964) for 3–10 min at room temperature. The dye solution was decanted off and the gels were washed with water.

Results

Time-course of β -glucuronidase response to gonadotrophin in subcellular fractions

The β -glucuronidase response to gonadotrophin in subcellular fractions up to 100 h after injection has been observed in kidneys of male BALB/C mice (Kato *et al.*, 1970). In the present paper the β -glucuronidase activity in subcellular fractions was measured for a longer period after the injection of gonadotrophin (Fig. 1). The increase in β -glucuronidase activity was first observed in the microsomal fraction, and the activity reached its maximum at 60 h. After that it decreased rapidly until 96 h, and then decreased gradually to the control value by 168 h after the injection. No change of β -glucuronidase

Fig. 1. Time-course of β -glucuronidase response to gonadotrophin in subcellular fractions

(●), Microsomal fraction; (○), lysosomal fraction. The cell-fractionation procedure and method of enzyme assay are described in the text.

Table 1. *Chemical and enzymic profiles of isolated microsomal subfractions from kidneys of control mice*

The microsomal subfractions were prepared as described in the Experimental section. The results are given as means $\pm$ s.d. for the numbers of experiments in parentheses. The values for rat and rabbit liver are taken from Dallner (1963) and Ito & Sato (1969) respectively.

Tissue and fraction	Protein (% of total)	RNA (μ g/mg of protein)	Phospholipid (μ g/mg of protein)	β -Glucuronidase (nmol/min per mg of protein)	Acid phosphatase (nmol/min per mg of protein)
Mouse kidney					
Rough microsomal	46 $\pm$ 4 (6)	164 $\pm$ 11 (4)	287 $\pm$ 15 (4)	1.0 $\pm$ 0.2 (3)	11 $\pm$ 2 (6)
1.3M-Sucrose	21 $\pm$ 2 (6)	71 $\pm$ 4 (4)	356 $\pm$ 30 (4)	1.4 $\pm$ 0.5 (3)	36 $\pm$ 3 (6)
Smooth microsomal	33 $\pm$ 4 (6)	22 $\pm$ 2 (4)	420 $\pm$ 27 (4)	2.3 $\pm$ 0.2 (3)	56 $\pm$ 5 (6)
Rat liver					
Rough microsomal		330	160		
Smooth microsomal		50	370		
Rabbit liver					
Rough microsomal		277	460		
Smooth microsomal		32	713		

activity in the lysosomal fraction was observed for the first 36h; after that it increased at a rate parallel to that of the microsomal β -glucuronidase and reached the maximum at 72h. The lysosomal activity then decreased at the same rate as the microsomal activity until 168h. The β -glucuronidase activity in the microsomal fraction reached its maximum at 60h, but the activity in the lysosomal fraction reached its maximum at 72h after the injection. The β -glucuronidase activity in the soluble fraction varied with time in essentially the same way as that of the lysosomal fraction and it might reflect the mechanical release of the enzyme from lysosomes during the course of tissue homogenization. This observation suggested that the site of the enzyme synthesis is probably microsomal and a likely pathway for the newly synthesized β -glucuronidase is from the endoplasmic reticulum to the lysosomes.

The effect of gonadotrophin on both acid phosphatase and glucose 6-phosphatase was also studied, but these enzyme activities did not fluctuate during the period described above.

Chemical and enzymic composition of microsomal subfractions

As little information has been reported on the subfractionation of kidney microsomal fraction, the method developed by Dallner (1963) for rat liver was applied. Table 1 shows the distribution of phospholipid, RNA and the activities of β -glucuronidase and acid phosphatase, compared with the values for rat and rabbit livers. The 1.3M-sucrose layer contained a considerable amount of protein with chemical and enzymic characteristics between those of the rough

and smooth microsomal fractions (cf. Dallner & Ernster, 1968). In agreement with the chemical analysis, electron-microscopic observations demonstrated that the rough and smooth subfractions were slightly cross-contaminated, and the 1.3M-sucrose layer contained both rough and smooth microsomal fractions.

The microsomal subfractions were also obtained from kidneys of gonadotrophin-injected mice, to see if gonadotrophin stimulation changes the chemical and enzymic characteristics of each microsomal subfraction. The subfractions showed patterns in the distribution of RNA, phospholipid and acid phosphatase activity similar to those of the controls.

Distribution of β -glucuronidase and acid phosphatase in submicrosomal fractions

Fig. 2 shows the distribution patterns of β -glucuronidase and acid phosphatase in submicrosomal fractions obtained from rough and smooth microsomal fractions by the deoxycholate treatment. The 1.3M-sucrose layer again contained characteristics of both the rough and smooth microsomal fractions. In both rough and smooth microsomal fractions about 70% of the total β -glucuronidase activity in each fraction was found in the cisternal contents and about 30% of the total activity was in the membrane fraction.

It is noteworthy that the smooth microsomal fraction contained much higher acid phosphatase activity than the rough counterpart, whereas in rat liver the reverse distribution between the rough and the smooth subfraction was reported (Glauman *et al.*, 1968).

Fig. 2. Distribution pattern of enzymes in submicrosomal fractions of the control kidneys

(a), β -Glucuronidase; (b), acid phosphatase. The submicrosomal fractions (contents and membranes) and rough and smooth microsomal fractions were prepared by the methods described in the text. Fractions are represented by their relative contents. A, B and C represent the 0.26%-deoxycholate-soluble, 0.5%-deoxycholate-soluble and 0.5%-deoxycholate-insoluble fractions respectively.

Time-course of submicrosomal β -glucuronidase response to gonadotrophin

Fig. 3 shows variation with time of the β -glucuronidase activity in each submicrosomal fraction of rough and smooth microsomal fractions after the injection of 2.5 i.u. of gonadotrophin. The marked increase in β -glucuronidase activity was first observed in rough microsomal membranes. At 24h after injection the increase in the activity was almost at the maximum and the elevated value was retained until 72h. Then the activity decreased until 120h, but at this time it was still higher than that of the control and this value was maintained until 168h after the injection. In the rough microsomal contents a striking increase in the enzyme activity was observed at 36h, and a rapid decrease was observed at 72h. The activity at 72h, which was still higher than that of the control, was maintained until 120h, and returned to the control value at 168h.

The increase in β -glucuronidase activity in the smooth submicrosomal fraction was delayed. The enzyme activity in the smooth microsomal fraction was almost unchanged until 24h after the injection, and an enhancement of the activity was first observed at 36h in smooth microsomal membranes, this value being maintained until 120h, then returning to the control value by 168h. The enzyme activity in the smooth microsomal contents increased sharply after 36h and reached its maximum at 72h. At this period, the enzyme activity in the rough microsomal contents decreased rapidly and the maximum enzyme activity was observed in the lysosomal fraction (Fig. 1). Subsequently the β -glucuronidase activity in the

smooth microsomal contents decreased rapidly, reaching the control value at 120h. The protein material in both rough and smooth microsomal contents varied with time in a fashion almost parallel to the enzyme activity, although not to such a great extent, the peaks being observed at 36h and 72h in rough and smooth microsomal contents respectively. The protein content in smooth microsomal membranes, however, remained unchanged throughout the experimental period. The protein content in the rough microsomal membrane fraction increased gradually, and it was only in this fraction that both protein content and β -glucuronidase activity persisted at an elevated value to the end of the experiment.

Effect of sonication on the release of microsomal β -glucuronidase

Sonication at 20000 Hz for 5 min released 45% of the total protein and 70% of the total β -glucuronidase from the microsomal fraction (Fig. 4). The same treatment solubilized both acid phosphatase and glucose 6-phosphatase to a smaller extent (about 30%) without any significant inactivation of enzymes. Treatment with sodium deoxycholate (0.26%) solubilized 40–45% of the total protein, and about 70% of the total β -glucuronidase and 30–35% of the total acid phosphatase from the microsomal fraction (Fig. 2). The amounts of protein and β -glucuronidase solubilized by sonication were thus almost equal to those solubilized by treatment with 0.26% deoxycholate. Although the rate of protein release by sonication decreased after 1 min, the enzyme liberation continued for up to 5 min. This delay of the

Fig. 3. Time-course of submicrosomal β -glucuronidase response to gonadotrophin

The rough and smooth microsomal fractions and the submicrosomal fractions were prepared by the methods described in the text. (a), Rough microsomal membranes; (b), rough microsomal contents; (c), smooth microsomal membranes; (d), smooth microsomal contents. β -Glucuronidase activity and protein contents of each submicrosomal fraction were measured and the percentage increase relative to the control was calculated. (●), β -Glucuronidase; (○), protein.

enzyme release suggested that intravesicular β -glucuronidase in the microsomal fraction is probably bound loosely to the membranes.

Digestion of microsomal fraction with trypsin

Digestion of kidney microsomal fraction with trypsin overnight at 0°C solubilized about 35% of the microsomal protein, as assessed by centrifugation at 105000g_{av.} for 60min. The amount of protein released was in good agreement with that found for rat liver microsomal fraction (Omura *et al.*, 1967). Inactivation of β -glucuronidase activity by trypsin digestion was not observed, and about 90% of the activity was found in the sedimentable fraction.

It is noteworthy that trypsin digestion released an amount of protein almost equivalent to that released by either 0.26% deoxycholate treatment or sonication, without any significant release of the β -glucuronidase activity. Although about 30% of microsomal acid phosphatase activity was released by either deoxycholate treatment or sonication, 10% of the enzyme activity was released by trypsin digestion, and 90% of the original activity was recovered in the sedimentable fraction.

The protein released by tryptic digestion seems to consist mainly of proteins located at the outside surface of the vesicles (Ito & Sato, 1969) and a part of the intravesicular protein that leaks out from the vesicles because of physiological changes of the

Fig. 4. Release of β -glucuronidase and intravesicular protein from the microsomal fraction after sonication

(●), Protein; (○), β -glucuronidase; (□), acid phosphatase; (■), glucose 6-phosphatase. Methods are described in the text.

membrane structure. The above results therefore suggested that β -glucuronidase and acid phosphatase are located in the inside of the vesicles.

Effect of incubation at 37°C on the release of microsomal β -glucuronidase

Incubation for 16h at 37°C released β -glucuronidase from the microsomal fraction and this phenomenon was dependent on the pH of the incubation mixture. About 35% of the total protein and 69% of the total β -glucuronidase was released after incubation of the microsomal fraction at pH5.0 (Table 2). A similar amount of protein was released at pH4.4, but only 18% of the enzyme was released. At pH5.5, the rate of solubilization of both protein (24%) and enzyme activity (18%) was lower. Thus β -glucuronidase was most favourably released at pH5.0. No enzyme activity was lost after incubation for 16h at 37°C at any pH value indicated in Table 2. Incubation for a shorter period, 2–3h, at any pH value (Table 2) did not solubilize more than 10% of either the total protein or the total β -glucuronidase activity from the microsomal fraction.

Table 2. Effect of incubation for 16h at 37°C and various pH values on the release of microsomal β -glucuronidase and microsomal protein

The incubation mixture, containing 2ml of microsomal suspension (5mg of protein/ml) in 0.25M-sucrose, 1ml of 0.4M-sodium acetate buffer, pH4.5, 0.3ml of 2M-NaCl, and adjusted to a final volume of 4ml with 0.25M-sucrose, was incubated for 16h at 37°C. After incubation the mixture was centrifuged at 105000g_{av} for 60min. Pellets were rinsed three times and resuspended in 0.25M-sucrose. β -Glucuronidase and protein in the supernatant and the pellets were determined as described in the text. The results are expressed as percentages of the total activity and the total protein found in the two fractions.

	Distribution (% of total)			
	pH 4.5	pH 5.0	pH 5.5	pH 6.0
Protein				
Soluble	35	35	24	19
Insoluble	65	65	76	81
β -Glucuronidase				
Soluble	18	69	18	13
Insoluble	82	31	82	87

Microsomal membranes obtained after sonication of the microsomal fraction released an additional 17% of the total protein and 5% of the total β -glucuronidase activity by incubation for 16h at 37°C, pH5.0 (Table 3). The two-step treatment of the intact microsomal fraction left 27% of the total β -glucuronidase in the sedimentable form. These results suggested that about 25% of the total β -glucuronidase is firmly bound to the microsomal membrane, and this fraction is termed the microsomal membrane β -glucuronidase hereafter. No differences in the solubilization experiment were observed between kidney microsomal fraction of the control and those of the gonadotrophin-stimulated mouse.

The incubation treatment inactivated the microsomal acid phosphatase activity. About 50% of the total activity was recovered in the precipitate after centrifugation of the incubated microsomal fraction at 105000g_{av} for 60min, but negligible amounts of activity were found in the supernatant.

Properties of β -glucuronidase from different cell organelles

Experiments were performed to determine the K_m , pH optimum, heat-stability and disc-electrophoretic mobility of β -glucuronidase in each of the three fractions. For this, the mitochondria-lysosome fraction was subjected to alternate freezing and thawing ten times and then was centrifuged at

Table 3. *Effect of various solubilization treatments on the release of microsomal β -glucuronidase and protein*

The microsomal suspensions were treated with trypsin, 0.26% sodium deoxycholate or by sonication as described in the text. For experimental details of incubation treatment at pH 5.0, see Table 2. For the two-step treatment given in the last column, the sedimentable fraction after sonication was further incubated for 16h at 37°C, pH 5.0. The results are expressed as percentages of the total activity and total protein found in sedimentable and non-sedimentable fractions.

	Distribution (% of total)				
	Trypsin digestion	0.26% Deoxycholate treatment	Sonication	Incubation at pH 5.0	Sonication, then incubation at pH 5.0
β -Glucuronidase					
Soluble	10	70	68	69	73
Insoluble	90	30	32	31	27
Protein					
Soluble	35	41	45	35	62
Insoluble	65	59	55	65	38

Table 4. *K_m values of *p*-nitrophenyl glucuronide for lysosomal and microsomal contents and microsomal membrane β -glucuronidases*

	K_m (mM)	
	Control	Gonadotrophin (10 i.u.)-stimulated
Lysosomes	15	15
Microsomal contents	15	15
Microsomal membranes	17	20

105000g_{av.} for 60min. The supernatant was used as the solubilized lysosomal β -glucuronidase. The microsomal β -glucuronidase of both the contents and the membranes was prepared by the ultrasonic disintegration method, except for the samples used for disc electrophoresis.

The K_m with *p*-nitrophenyl glucuronide as substrate showed no significant difference among the three β -glucuronidases (Table 4). Fig. 5 shows the pH optimum of each β -glucuronidase from the different sources. The β -glucuronidases of the microsomal contents and lysosomes had essentially the same pH-activity curves and only a single peak (pH 4.5–4.7) was observed. The microsomal membrane enzyme had its maximum activity between pH 4.2 and 4.5 with a shoulder at about pH 5.5. However, there were no significant differences in pH optimum between these three enzymes.

Paigen (1961) reported that the rate of heat-inactivation of mouse liver β -glucuronidase depends on the strain of mouse, and strain C3H/He enzyme is

Fig. 5. *Effect of pH on β -glucuronidase activity*

The lysosomal ($\circ$, $\bullet$) and microsomal (Δ , $\blacktriangle$) contents and the microsomal membrane ($\square$, $\blacksquare$) preparations were incubated for 60min at 37°C with 0.01 M-*p*-nitrophenyl glucuronide, in 0.1M-sodium acetate buffer ($\circ$, Δ , $\square$), or 0.05 M-sodium phosphate buffer ($\bullet$, $\blacktriangle$, $\blacksquare$). Enough NaCl was added to bring the ionic strength to 0.15 and the final pH was recorded.

inactivated at 71°C several times as rapidly as is strain DBA/2 enzyme. Heat-inactivation accordingly seems to be a useful test for characterizing the three enzymes. Fig. 6 shows the heat-inactivation kinetics of the three enzymes at 70°C and at different pH

Fig. 6. Heat inactivation of β -glucuronidase

(a), Lysosomal contents; (b), microsomal contents; (c), microsomal membrane. The enzyme activity is plotted as a function of the duration of exposure to heating at 70°C. Activities are expressed as percentages of the initial activity. (○), Sodium acetate buffer, pH 4.5; (△), sodium phosphate buffer, pH 6.7; (▲), sodium phosphate buffer, pH 7.0; (□), tris-HCl buffer, pH 7.0.

values (4.5, 6.7 and 7.0). In the presence of either sodium acetate buffer, pH 4.5, or tris-HCl buffer, pH 7.0, most activity of the three enzymes survived after 60 min of incubation at 70°C. However, the replacement of tris buffer, pH 7.0, with sodium phosphate buffer, pH 7.0, caused a rapid loss of the activity of the three enzymes. Except for the slight activation of the membrane enzyme after a short period of incubation, there was no significant difference between the three enzymes in the heat-inactivation test.

The mobility of the three enzymes was compared by electrophoresis in the polyacrylamide-gel system of Davis (1964) (Fig. 7). The microsomal β -glucuronidases of both contents and membranes were prepared by the deoxycholate treatment because the membrane enzyme was solubilized by incubation of the 0.5% deoxycholate solution of the membranes at 0°C to such an extent that approx. 70% of the total activity was released after 7 days. The solubilized membrane β -glucuronidase after 7 days had an electrophoretic pattern identical with that of the enzyme released after overnight incubation. Therefore, the sample for disc electrophoresis shown in Fig. 7 was prepared by the shorter period of incubation.

The lysosomal enzyme had a single activity band. The microsomal enzymes of both the contents and the membranes gave two bands located very close to each other. In addition, their mobilities were also close to that of the lysosomal enzyme. When the microsomal enzymes of both contents and membranes were run together, the same two bands were obtained. The results indicated that the β -glucuronidase located in the microsomal contents is probably the same enzyme as that in membranes. When the enzyme of each submicrosomal fraction was run together with the lysosomal enzyme, two bands located close to each other were observed. The results indicated that one of the two activity bands of the microsomal enzymes was superimposed on that of the lysosomal enzyme, but which band has the same mobility as that of the lysosomal enzyme could not be decided. Although a second activity band was observed with microsomal enzymes, disc electrophoresis revealed that the lysosomes contain a β -glucuronidase that is probably identical with one of the microsomal enzymes. There were no differences in the enzyme property between gonadotrophin-stimulated mice and the controls.

In this experiment liver β -glucuronidases were also

Fig. 7. Disc-gel electrophoresis of kidney and liver β -glucuronidase

The samples of solubilized enzyme from lysosomes (a), microsomal contents (b) and microsomal membranes (c) were prepared as described in the text. The samples were subjected to electrophoresis by the method of Davis (1964). After 4 h at 5 mA/gel (10 mm $\times$ 60 mm), the gels were stained for β -glucuronidase activity as described in the text.

tested. The lysosomal enzymes of both kidney and liver showed a single band of the same mobility. The liver submicrosomal enzymes had the same two bands as those of the kidney enzymes, and two additional faint bands were observed with the microsomal contents.

Discussion

The studies of Caro & Palade (1964) on the secretory cycle of the exocrine pancreas initiated speculation on the formation of other secretory granules as well as primary lysosomes. The basic assumption is that lysosomal enzymes are synthesized in the rough endoplasmic reticulum and enter the cisternae of this structure, and then are transported to the Golgi complex via the smooth endoplasmic reticulum. The Golgi complex presumably functions in packaging newly synthesized enzymes into a heterophagic vacuole and initiates the digestive process. The above formulation is specu-

lative and based on static morphological studies. The site for biosynthesis of lysosomal enzymes and the path of the flow of the newly synthesized proteins from rough endoplasmic reticulum through smooth endoplasmic reticulum into the Golgi complex are still biochemically uncertain.

Our findings, based on the time-course of subcellular and submicrosomal β -glucuronidase response to gonadotrophin, suggested that the site of the enzyme synthesis is probably the membrane of the rough microsomal fraction. The newly synthesized enzyme at the site of membrane-bound polyribosomes enters the cisternae of the rough endoplasmic reticulum, and is then transported through the smooth endoplasmic reticulum into lysosomes. At present, because cell-fractionation techniques cannot separate the mouse renal Golgi complex, the role of this cell organelle is still uncertain. Further, because the increased β -glucuronidase activity in the lysosomal fraction declines rapidly at the same rate as that in the microsomal fraction (Fig. 1), it is not known whether the β -glucuronidase stimulated by gonadotrophin is contained in the normal lysosomes or in some special vesicles budding off from the smooth endoplasmic reticulum without other acid hydrolases.

It is noteworthy that the enhancement of β -glucuronidase activity in the smooth microsomal membranes occurs before that in the smooth microsomal contents. This suggests that the newly synthesized enzyme, which was released from the membrane of rough endoplasmic reticulum and then migrated to the smooth endoplasmic reticulum, was again associated with the membrane and afterwards accumulated in the cisternae of the latter structure. According to Schnaitman (1969), although rough- and smooth-microsomal membrane fractions contained a number of similar proteins, some proteins were observed that were unique to each fraction. Dallner *et al.* (1968) have also suggested that the endoplasmic reticulum from which these fractions are derived is heterogeneous. Therefore the above result could be attributed to the difference between membrane proteins of rough and smooth endoplasmic reticulum.

It has been shown (de Duve *et al.*, 1955) by tissue-fractionation studies that rat liver β -glucuronidase has a dual localization, being contained in the lysosomal fraction and in the microsomal fraction. The latter fraction contains about 35% of the total β -glucuronidase activity present in homogenates of rat liver. These results were confirmed (Fishman *et al.*, 1967) by cytochemical techniques. Mouse kidneys also possess 20–25% of the total β -glucuronidase in the microsomal fraction. Treatment of rough and smooth microsomal fractions of mouse kidney with deoxycholate or by sonication showed that about 70% of the β -glucuronidase activity was in the

cisternal contents and about 30% of that was in the membrane fraction (Table 3). The specific activities of β -glucuronidase in rough and smooth microsomal fractions were 1.0 and 2.3, respectively (Table 1). The smooth microsomal fraction contained much higher acid phosphatase activity than the rough counterpart. The specific activity of each fraction was 11 and 56, respectively (Table 1). Treatment of the smooth microsomal fraction with deoxycholate or by sonication showed that 30–35% of the acid phosphatase activity was in the cisternal contents and about 55% of that was in the membrane fraction (Fig. 2). Acid phosphatase seems to be more firmly bound to the membrane of smooth endoplasmic reticulum than β -glucuronidase is.

About 70% of the β -glucuronidase activity bound to the microsomal membrane fraction was released by the incubation of the membrane in 0.5% deoxycholate at 0°C for 7 days. The solubilized membrane β -glucuronidase did not differ significantly from the β -glucuronidase in the microsomal contents with respect to K_m , pH optimum, heat-stability and disc-gel-electrophoretic mobility. Also there were no significant differences between the microsomal β -glucuronidase and the lysosomal β -glucuronidase with respect to K_m , pH optimum and heat-stability. The disc-gel-electrophoretic pattern of each enzyme of microsomal and lysosomal origin, however, was different. The microsomal enzyme had two activity bands very close to each other, whereas the lysosomal enzyme had a single activity band. As one of the activity bands of the microsomal enzyme was superimposed on that of the lysosomal enzyme when both enzymes were run together, the lysosomes probably contain a β -glucuronidase identical with one of the microsomal enzymes. However, the relation between the two enzymes in the microsomal fraction is still uncertain. Whether one of them is located in the endoplasmic reticulum and not in the lysosomes or whether these enzymes have different carbohydrate

chains but the same protein moiety, is of considerable interest, but is a problem that remains to be solved.

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