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Incorporation of [^{14}C]Glucosamine and [^{14}C]Leucine into Mouse Kidney β-Glucuronidase Induced by Gonadotrophin

By KEITARO KATO,* HIROYUKI IDE,† TSURANOBU SHIRAHAMA‡ and WILLIAM H. FISHMAN
Department of Pathology (Oncology), Tufts University School of Medicine and the Cancer Research Department, New England Medical Center Hospitals, Boston, Mass. 02111, *U.S.A.*

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Male BALB/C mice were injected intraperitoneally with 2.5 i.u. of gonadotrophin. After the injection, increase of β-glucuronidase activity was first observed in the microsomal fraction. By 36h 45–50% of the total homogenate activity was found in the microsomal fraction compared with 20–25% in the control microsomal fraction. From 36 to 80h not only microsomal β-glucuronidase but also lysosomal β-glucuronidase increased progressively. After 69h stimulation with 2.5 i.u. of gonadotrophin, D-[1-^{14}C]glucosamine or L-[U-^{14}C]leucine was injected intraperitoneally. After a further 3h the kidneys were homogenized and five particulate fractions were prepared by differential centrifugation. The β-glucuronidase in the microsomal and lysosomal fractions was released respectively by ultrasonication and by freezing and thawing treatment. The enzyme was purified by organic-solvent precipitation and by sucrose-density-gradient centrifugation. The results demonstrated the incorporation of these two labels into the mouse renal β-glucuronidase. The microsomal β-glucuronidase was much more radioactive than the lysosomal enzyme and approx. 80% of the newly synthesized enzyme appeared in microsomes and approx. 20% of that was found in lysosomes at this period. These results suggest that the mouse renal β-glucuronidase is a glycoprotein and that the newly synthesized enzyme is transported from endoplasmic reticulum to lysosomes.

The increase in renal β-glucuronidase activity after androgen stimulation is due to a synthesis of this particular protein *de novo*, on the basis of [^{14}C]-glycine-incorporation experiments of Pettengill & Fishman (1962*b*) and of the actinomycin D inhibition results of Frieden, Harper, Chin & Fishman (1964). Moreover, the β-glucuronidase response is specific to androgenic hormones and is independent of the factors that govern the activity of a number of other hydrolases in mouse kidney.

According to the reports of Plapp & Cole (1966, 1967), β-glucuronidase purified from calf liver is a glycoprotein and contains 3–6% glucosamine and hexose.

In designing the present study, it has been assumed that mouse renal β-glucuronidase is also a glycoprotein, although no direct evidence on this point was available. If [^{14}C]glucosamine were to be incorporated into β-glucuronidase synthesized as a consequence of androgen action, such a result would not only prove the glycoprotein nature of the enzyme but in turn would provide a reason to employ β-glucuronidase as a marker for glycoprotein synthesis. Finally, such an experiment would afford an opportunity of tracing the path of a typical lysosomal hydrolase from the organelle site of its genesis, the endoplasmic reticulum, to its destination, the lysosome organelle.

In the experiments described below, mice were subjected to a single potent stimulation by testicular androgen produced with an injection of gonadotrophin. At the height of the renal β-glucuronidase response (after 72h), measurements were made of the extent of incorporation of these two labels into β-glucuronidase. Further, the results so obtained are consistent with the intracellular transport of the newly synthesized enzyme from endoplasmic reticulum to lysosomes.

* Present address: Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan.
† Present address: Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan.
‡ Present address: Department of Medicine, Boston University School of Medicine, Boston, Mass. 02111, U.S.A.

METHODS

Tissue fractionation. Each subcellular fraction was prepared from the kidneys of male BALB/C mice by the slight modification of the method described by Ide & Fishman (1969), by using the Sorvall refrigerated centrifuge and the Spinco model L preparative centrifuge. A tissue homogenate (15% w/v) in 0.25M-sucrose containing 0.05M-sodium phosphate buffer, pH 7.4, was centrifuged at $150g_{max.}$ for 2min. The sediment was discarded, then five particulate fractions and soluble fraction were obtained by centrifuging the supernatant successively for 2min at $1950g_{max.}$ (fraction A, unbroken cells and nuclei), 2min at $7170g_{max.}$ (fraction B, mitochondrial–lysosomal fraction), 5min at $17300g_{max.}$ (fraction C, light mitochondrial–lysosomal fraction), 60min at $41225g_{av.}$ (fraction D, microsomal fraction), and 90min at $105000g_{av.}$ (fraction E, light microsomal fraction). The remaining supernatant was used for assay of the enzymes in the unsedimentable fraction.

Determination of β-glucuronidase activity. β-Glucuronidase was determined by the method of Fishman (1965) with phenolphthalein glucuronide as substrate. Specific activity is expressed as nmol of substrate hydrolysed/min per mg of protein, 1 unit being the amount of enzyme catalysing hydrolysis of 1nmol of substrate/min.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

Treatment with ultrasonic oscillation. The kidney microsome pellet (fraction D) was first suspended in 0.25M-sucrose and adjusted to a concentration of 1mg of protein/ml. Samples (10ml) were treated for different times (0.5, 1, 3 and 5min) with a MSE ultrasonic disintegrator operating at 20000Hz. All suspensions obtained by ultrasonic oscillation were centrifuged at $105000g_{av.}$ for 1h to yield tightly packed pellets consisting of ribosomes and membranes, and supernatants that represent the microsomal contents together with membrane components that were no longer sedimentable.

Variation with time of subcellular β-glucuronidase response to gonadotrophin. Male BALB/C mice, about 3 months old and weighing 27±2g, were arranged in eight groups of six animals. The mice of seven groups were injected intraperitoneally with 2.5 i.u. of human chorionic gonadotrophin (Squibb, New York, N.Y., U.S.A.) in 0.85% NaCl, pH 7.5, and killed at intervals of 12h until 96h after the injection of gonadotrophin, one group injected only with 0.85% NaCl serving as a control for the other groups. Cell fractionation was carried out as described above on the kidneys, which weighed about 2.5g per group. β-Glucuronidase activity of each subcellular fraction was measured and the percentage increase in activity relative to the control was calculated.

Purification of β-glucuronidase from mouse kidney microsomes and lysosomes. Eighteen male BALB/C mice were injected intraperitoneally with 2.5 i.u. of gonadotrophin as described above. The mice were killed 72h after injection and about 6g of kidneys was obtained. From the fractionation of the homogenate of the tissue in the sucrose–phosphate buffer, fractions B and C were collected, pooled and used as a lysosomal fraction. The combined fraction was suspended in 13ml of 0.25M-sucrose–0.05M-phosphate buffer, pH 7.4 (20mg of protein/ml) and subjected to alternate freezing and thawing ten times. The resulting suspension was centrifuged at $105000g_{av.}$ for 1h, yielding a supernatant solution of soluble lysosomal β-glucuronidase. Fraction D (microsomes) was suspended in 20ml of the same buffer (5–6mg of protein/ml) and treated for 3min with an MSE ultrasonic disintegrator operating at 20000Hz. The suspension obtained by ultrasonic oscillation was centrifuged at $105000g_{av.}$ for 1h and the supernatant was separated. This contained soluble microsomal β-glucuronidase as defined by Ide & Fishman (1969).

Each extract of microsomal or lysosomal β-glucuronidase was precipitated as described by Pettengill & Fishman (1962*a*) by the addition of 0.5vol. of ice-cold organic solvent mixture (ethanol–acetone–ether, 15:4:1, by vol.) and cooled in ice for 20min. The mixture was centrifuged at $17300g_{max.}$ for 20min and the precipitate was discarded. The supernatant was treated again with an equal volume of the organic-solvent mixture described above and cooled in ice for 20min. The mixture was centrifuged at $17300g_{max.}$ for 20min and the supernatant was poured off and discarded. The precipitate was dissolved in 2ml of 0.025M-sodium phosphate buffer, pH 7.4, and insoluble material was removed by centrifugation at $105000g_{av.}$ for 1h. The above organic-solvent treatment was repeated twice for the freezing-and-thawing extract of the lysosomal enzyme. However, the ultrasonic extract of the microsomal enzyme was too low in protein to permit the second purification with organic solvents.

Next, 1ml of the enzyme solution (0.8–1.0mg of protein) obtained by the organic-solvent treatment was layered on a linear sucrose density gradient (32ml; 5–20% sucrose in 0.025M-sodium phosphate buffer, pH 7.4) and the tubes were centrifuged at $63500g_{av.}$ (Spinco no. SW 25.1 rotor) for 16h. At the end of centrifugation, fractions (approx. 1ml) were collected from the bottom of the tubes and were assayed for β-glucuronidase activity and protein. Fraction 23 had the highest specific activity of β-glucuronidase whether prepared from microsomes or from lysosomes.

Incorporation of D-[^{14}C]*glucosamine and* L-[^{14}C]*leucine into microsomal and lysosomal β-glucuronidase.* Male BALB/C mice, about 2 months old and weighing 20±2g, were arranged in two groups each of 18 animals. The mice of both groups were injected intraperitoneally with 2.5 i.u. of gonadotrophin in 0.85% NaCl, pH 7.5. At 69h after injection, 8μCi of D-[1-^{14}C]glucosamine or 4μCi of L-[U-^{14}C]leucine (New England Nuclear Corp., Boston, Mass., U.S.A.) in 0.85% NaCl was injected intraperitoneally into the mice of the two groups respectively. The mice were killed exactly 3h after the injection of radioactive materials (72h after the injection of gonadotrophin). Cell fractionation and purification of microsomal and lysosomal β-glucuronidase were carried out as described above. After sucrose-density-gradient centrifugation, radioactivity of each fractionated tube was determined as follows. Nuclear–Chicago Protein-Solubilizing Reagent (NCS; 0.5ml) was incubated with 0.1ml of specimen at 40°C and then the clear solution was mixed with 10ml of scintillation fluid prepared as described by Wannemacher, Banks & Wunner (1965). Radioactivity was measured in a Nuclear–Chicago mark I scintillation counter.

RESULTS

Time-course of subcellular β-glucuronidase response to gonadotrophin. The early effects of gonadotrophin were studied after the single injection of 2.5 i.u. of gonadotrophin at intervals of 12h until 96h (Fig. 1). The increase in β-glucuronidase activity was first observed in fraction D (microsomes). At 36h 45–50% of the total homogenate activity was found in the microsomal fraction, compared with 20–25% in the microsomal fraction of kidneys of animals injected only with 0.85% sodium chloride. By contrast, β-glucuronidase activity was almost unchanged in all the other fractions until 36h after injection. After this period, not only microsomal β-glucuronidase activity but also the lysosomal activity increased progressively with time until 80h. These results favour the idea that the enzyme newly synthesized in the endoplasmic reticulum after the injection of gonadotrophin makes its appearance in the lysosomes by 36h.

Effect of ultrasonic oscillation on the release of microsomal β-glucuronidase. To separate microsomal contents from their membranes and associated ribosomes, the microsomal fraction was treated by ultrasonic oscillation. Ultrasonic extracts of the microsomes contain intravesicular material. As shown in Fig. 2, sonication at 20000Hz for 3min released 37% of the total protein and 75% of the total β-glucuronidase from the microsomes. A similar result was obtained by the procedure of Dallner, Siekevitz & Palade (1966), with 0.26% deoxycholate (Kato, Hirohata & Muta, 1968).

In the ultrasonic-oscillation treatment, release of the protein reached a plateau at 1min, at which time 45% of the enzyme was solubilized. Release of the enzyme increased to 89% at 5min, i.e. 11% of the enzyme still remained in the membrane fraction at this period. Enzyme activation by unfolding might have occurred from 1 to 5min, as the sum of the supernatant and sediment activities exceeds 100% of that originally present. These results suggest that microsomal β-glucuronidase is associated with both the intravesicular protein and the vesicular membranes.

Incorporation of D-[^{14}C]*glucosamine and* L-[^{14}C]*leucine into microsomal and lysosomal β-glucuronidase.* Experiments on D-[^{14}C]glucosamine and L-[^{14}C]leucine incorporation are shown in Figs. 3–6 and in Tables 1 and 2. It is apparent that 3h after injection of tracer, the microsomal β-glucuronidase is more radioactive than the lysosomal enzyme.

Table 1 shows a summary of the results of experiments on incorporation into the β-glucuronidase (fraction 23) released from microsomes and

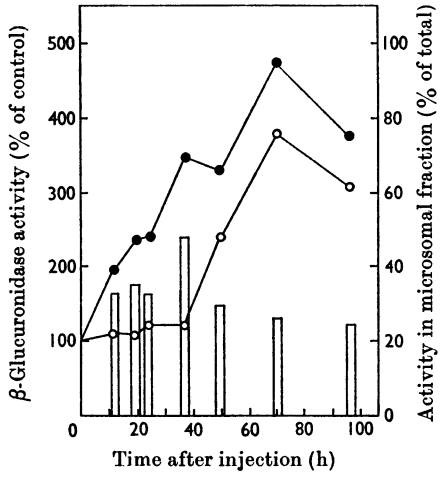

Fig. 1. Time-course of subcellular β-glucuronidase response to gonadotrophin. Fraction D was used as a microsomal fraction (●). Fractions B and C combined were used as a lysosomal fraction (○). Columns show percentage of total homogenate activity in the microsomal fraction at each period. The cell fractionation procedure and a method of enzyme assay are described in the text.

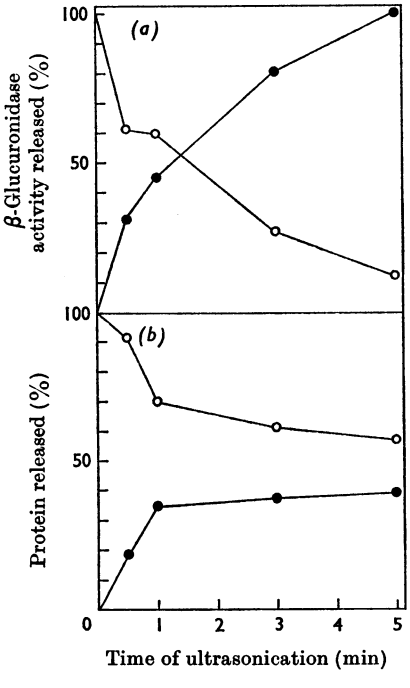

Fig. 2. Release of β-glucuronidase (*a*) and intravesicular protein (*b*) from microsomes after treatment with ultrasonic oscillation. (●), Supernatant; (○), sedimented material. Methods are described in the text.

Table 1. *Incorporation of* D-[^{14}C]*glucosamine and* L-[^{14}C]*leucine into β-glucuronidase (fraction 23) of kidney microsomes and lysosomes*

Tracer	Microsomes		Lysosomes	
	Specific activity (units/mg)	Radioactivity of β-glucuronidase (d.p.m./mg)	Specific activity (units/mg)	Radioactivity of β-glucuronidase (d.p.m./mg)
D-[^{14}C]Glucosamine	1939	8600	2934	1990
L-[^{14}C]Leucine	2175	9850	3960	2710

Table 2. *Purification of mouse kidney β-glucuronidase and incorporation of* D-[^{14}C]*glucosamine at each stage of the purification*

Description of step	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Radioactivity (d.p.m./mg)
Homogenate	34	18.50	7.2	4690
Microsomes				
Fraction D	21	5.28	9.8	4700
Ultrasonic supernatant	18.4	1.28	29.0	5260
Ultrasonic precipitate	5	18.50	2.4	3120
Organic-solvent precipitation	2	0.87	198	5580
Sucrose-density-gradient centrifugation		(see Fig. 3 and Table 1)		
Lysosomes				
Fractions B and C	15	13.48	9.1	2990
Freezing-and-thawing supernatant	11	3.44	26.3	3220
Organic-solvent precipitation	2	0.81	339	2480
Sucrose-density-gradient centrifugation		(see Fig. 4 and Table 1)		

lysosomes. Detailed results for the stepwise purification that preceded the final preparation of fraction 23 are illustrated in Table 2 for the D-[^{14}C]-glucosamine experiment only. The plots of specific activity and radioactivity versus tube number in the sucrose-density-gradient studies in Figs. 3–6 show clearly the purity of the enzyme in tube 23, the correspondence of the enzyme peak with the peak of radioactivity (whether from glucosamine or leucine) in microsomal β-glucuronidase and the relative lack of radioactive protein in the lysosomal β-glucuronidase.

In our tissue-fractionation technique, the lysosomal fraction usually contains heavy microsomes. Since the specific radioactivity of microsomal β-glucuronidase was four times that of the lysosomal enzyme, it is desirable to estimate how much microsomal β-glucuronidase contaminates the lysosomal β-glucuronidase. This was determined from measurement of its glucose 6-phosphatase activity. On the other hand, the degree of contamination of the microsomal fraction by lysosomes can be evaluated from the β-glucuronidase released by the freezing-and-thawing treatment.

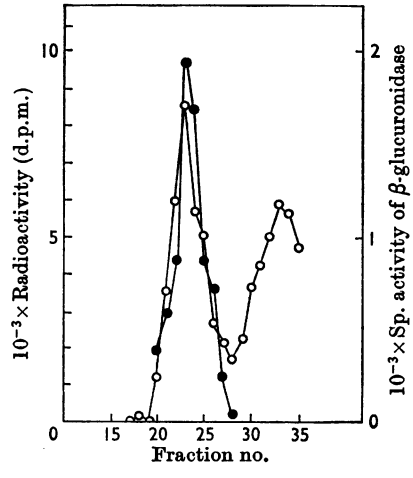

Fig. 3. Sucrose-density-gradient profile (5–20% sucrose; sedimentation is from left to right) for microsomal β-glucuronidase prepared from kidneys of mice injected with D-[^{14}C]glucosamine. For Figs. 3–6, (●) represents β-glucuronidase specific activity (nmol/min per mg of protein) and (○) radioactivity (c.p.m.).

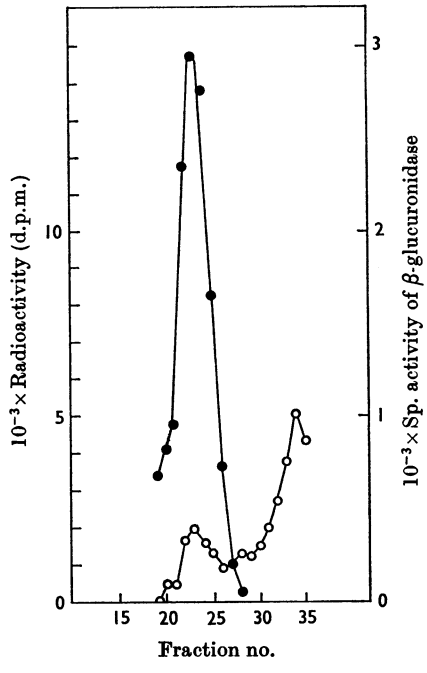

Fig. 4. Sucrose-density-gradient profile of lysosomal β-glucuronidase (label, D-[^{14}C]glucosamine).

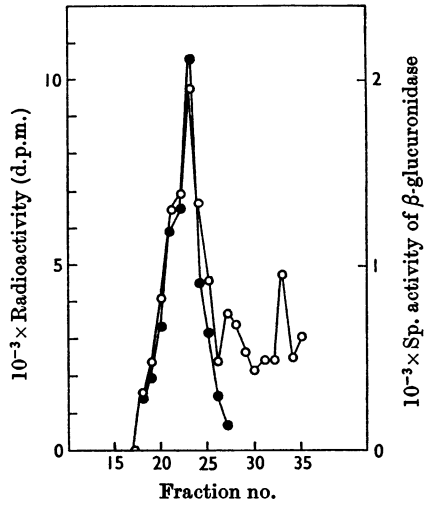

Fig. 5. Sucrose-density-gradient profile (5–20% sucrose; sedimentation is from left to right) for microsomal β-glucuronidase prepared from kidneys of mice injected with [^{14}C]leucine.

According to Ide & Fishman (1969), only 5–8% of the total β-glucuronidase activity was released from the microsomes after freezing and thawing ten times. Computation based on the above results shows that the maximum contaminating radioactivity of microsomal β-glucuronidase in the lysosomal enzyme is approx. 300 d.p.m. in the 1990 d.p.m./mg for D-[^{14}C]glucosamine incorporation and approx. 400 d.p.m. in the 2710 d.p.m./mg for L-[^{14}C]leucine incorporation.

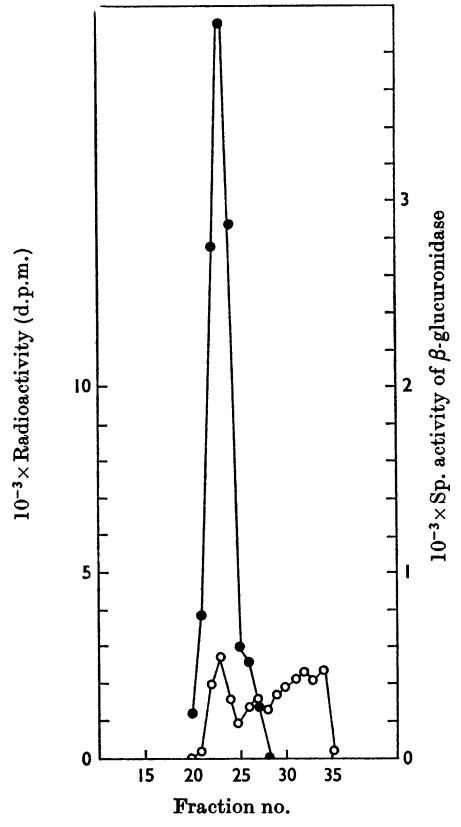

Fig. 6. Sucrose-density-gradient profile for lysosomal β-glucuronidase (label, [^{14}C]leucine).

From the specific radioactivity (d.p.m./enzyme unit) of the β-glucuronidase in microsomal and lysosomal fractions, one can also calculate roughly the relative amount of the newly synthesized enzyme in each fraction. Thus, approx. 80% of the newly synthesized enzyme is in microsomes and approx. 20% is in lysosomes at 3h after the injection of D-[^{14}C]glucosamine or L-[^{14}C]leucine. The microsomal vesicles derived from the endoplasmic reticulum are the sites of first incorporation into enzyme of radioactive glucosamine and leucine and the lower incorporation of these labels into β-glucuronidase of lysosomes suggests that the direction of enzyme movement is from endoplasmic reticulum to lysosomes.

DISCUSSION

The extent of labelling of the renal β-glucuronidase by D-[^{14}C]glucosamine and L-[^{14}C]leucine in gonadotrophin-stimulated mice permits the conclusion that a specific glycoprotein with the catalytic activity of β-glucuronidase has been biosynthesized.

The microsomal β-glucuronidase increases almost linearly to a peak at 80h whereas no change in the lysosomal β-glucuronidase occurs for the first 36h, after which it increases to an extent that parallels the microsomal β-glucuronidase. At 36h over 45–50% of the total activity is in the microsomal fraction. The unstimulated mice normally possess only 20–25% of the total β-glucuronidase in the microsomal fraction. The choice of the 72h interval was made because this provided almost the largest amount of enzyme activity at a time when the rate of increase of the enzyme activity was being maintained in each of the microsomal and lysosomal fractions.

The compositions of fractions A, B, C, D and E were evaluated from the electron micrographs and from the results for marker enzymes reported by Ide & Fishman (1969). Freezing-and-thawing treatment released 70% of the β-glucuronidase from fractions B and C (rich in lysosomes) into a non-sedimentable form. On the other hand, only 5–8% of the total β-glucuronidase activity was released from fraction D after the same treatment. Ultrasonic treatment was required to convert the membrane-bound β-glucuronidase into a non-sedimentable form in the microsomes in fraction D. After ultrasonication at 20000Hz for 3min, 75% of the enzyme was non-sedimentable and 25% was associated with the membrane fraction, the intravesicular β-glucuronidase having been released after only 1min. The β-glucuronidase released from microsomes or lysosomes possessed roughly the same molecular weight, as judged by the sucrose-density-gradient centrifugation experiments, and had similar properties (Ide & Fishman, 1969).

Significant labelling with D-[^{14}C]glucosamine occurred in the membrane precipitate prepared by centrifuging the ultrasonicated preparation from microsomes. Of the radioactivity 70% was in this fraction, and the other 30% was in the supernatant, presumably derived from the cisternal contents. The association of β-glucuronidase with this precipitate, which can be presumed to be rich in microsomal membranes, is substantial (25% of the total activity of the intact microsomes). The incorporation of newly formed β-glucuronidase into this fraction is significant, since its specific activity (2.4) is considerably higher than that (0.6) established for the corresponding fraction prepared from unstimulated mice. In the experiment of Kato *et al.* (1968) in which the technique of Dallner *et al.* (1966) was employed to separate out the submicrosomal fractions with deoxycholate, a similar enhancement in the specific activity of the membrane fraction was observed in strain DDD mice. This evidence of enrichment of cytoplasmic membranes with β-glucuronidase is consistent with the acid-hydrolase membrane hypothesis of Fishman, Goldman & DeLellis (1967).

With regard to the purity of the lysosomal β-glucuronidase in fraction 23 of the stimulated mouse kidney, its specific activity (4000nmol/min per mg) compares well with the specific activity of the best mouse kidney β-glucuronidase purified from the kidney homogenates by an independent method of purification (4200) (Pettengill & Fishman, 1962*a*) but which included the solvent-precipitation technique. The stepwise increase in the concentration of the acetone–alcohol–ether mixture, which succeeded initially in removing much non-β-glucuronidase protein from the mixture has been of particular value. Most of the enzyme was precipitated by the second addition of organic solvent. Contributing even more to the final purification has been the use of sucrose-density-gradient centrifugation. It is noteworthy that the β-glucuronidase released from lysosomes and microsomes in each case was concentrated in fraction 23 of the sucrose density gradient. These enzymes from different subcellular origins might have the same molecular weight once they have been dissociated from their membrane positions.

From the specific radioactivity of the β-glucuronidase in microsomal and lysosomal fractions, it seems reasonable to suggest that in 3h about 20% of the newly synthesized enzyme was transported from endoplasmic reticulum to the lysosomes. This suggestion is supported by the experiments in which during the first 36h of the stimulation progressive uninterrupted increase in specific activity of microsomal β-glucuronidase was observed before an increase in specific activity of the lysosomal enzyme was detected.

Whether the β-glucuronidase stimulated by androgen is contained in the normal lysosomes or in some special vesicles budding off from the Golgi apparatus or smooth endoplasmic reticulum without other acid hydrolases is still unknown. It is possible that the response to androgen by the mouse renal β-glucuronidase may permit the route of intracellular transport of a lysosomal enzyme to be traced.

Finally, the incorporation of [^{14}C]leucine paralleled that of [^{14}C]glucosamine, suggesting that the synthesis of the polypeptide and carbohydrate portions of the molecule was contemporaneous. In future work, it would be desirable to recover [^{14}C]-glucosamine from the most active β-glucuronidase fraction to provide direct evidence of incorporation.

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