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STUDIES ON A SOLUBILIZED OESTRIOL 16α-GLUCURONYLTRANSFERASE FROM HUMAN LIVER MICROSOMES

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SUMMARY

Treatment of lyophilized human liver microsomes with deoxycholate led to "solubilization" of oestriol 16α-glucuronyltransferase. The enzymic activity was stable in the presence of dithiothreitol and EDTA. A five fold enrichment of enzyme activity was obtained after filtration of the "solubilized" enzyme through Sepharose 4B. Phospholipase C reduced the enzyme activity by 70%, lysolecithin restored the activity to 75%. Phosphatidylcholine and sphingomyelin were found to be the major phospholipids in the partially purified oestriol 16α-glucuronyltransferase. From the ratio of void volume to elution volume, the apparent molecular weight of the active fraction was found to be approximately two million. These results suggest that the oestriol 16α-glucuronyltransferase "solubilized" from human liver microsomes may be a lipid-protein complex.

INTRODUCTION

THE CONJUGATION of steroid hormones with glucuronic acid is catalyzed by UDP-glucuronyltransferases (UDP-glucuronate glucuronyltransferase; EC 2.4.1.17); these enzymes are considered to be present primarily in the microsomal fraction of mammalian liver. Recently, we reported the presence of an oestriol 16α-glucuronyltransferase in the cytosol fraction of human liver[1]. From a consideration of several possibilities, it appeared that this enzymic activity was associated with extremely fine fragments from the endoplasmic reticulum. In preliminary studies it was observed that the microsomal fraction of human liver conjugated the 16α-hydroxyl group of oestriol with glucuronic acid.

There is evidence that UDP-glucuronyltransferases originate from membranes of microsomes and may be solubilized by digestion with snake venom or treatment with detergents. Attempts to solubilize the microsomal enzyme are intimately connected with partial or total loss of enzymic activity. Several reports in recent literature deal with experiments in which solubilization has been claimed to be achieved; the "solubilized" enzyme has been purified to some extent. The enzyme activity in all instances has been tested with a wide variety of non-steroidal substrates[2–7]. However, studies on solubilization of *steroid* glucuronyltransferases have received very little attention. Since it is known that several different glucuronyltransferases exist, it becomes all the more essential to obtain particulate glucuronyltransferases in a "soluble" form, in order to investigate the properties of the enzymes and to study their role in the enterohepatic circulation of oestrogens.

This paper describes experiments in which a glucuronyltransferase, conjugating the 16α-hydroxyl group of oestriol, has been "solubilized" from the microsomal fraction of human liver by treatment with sodium deoxycholate. Attempts have been made to purify the "solubilized" enzyme and to study the physical properties of the partially purified enzyme.

MATERIALS

Buffer solutions were prepared as described previously [1].

[4-^{14}C] Oestriol (1,3,5(10)-estratriene-3,16α,17β-triol), S. A. 51·4 mCi/mmol, was obtained from The Radiochemical Centre, Amersham, Bucks, England.

UDP-glucuronic acid disodium salt was generously donated by Prof. H. U. Bergmeyer, Boehringer Mannheim GmbH, Biochemische Abteilung, Tutzing, Germany. Trypsin (lyophilized) was purchased from Boehringer Mannheim GmbH, Germany. Phospholipase C (Cl. welchii) was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Phospholipids were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England. All other chemicals were obtained from commercial sources and used without further purification. Sepharose 4B and Sepharose 2B were purchased from Deutsche Pharmacia, Frankfurt/Main, Germany. Solvents were analytical grade; wherever necessary, solvents were redistilled before use.

EXPERIMENTAL

The liver was obtained from the surgery unit during a kidney transplantation. The subject was a 25 year-old male who had taken an overdose of barbiturate and had been given dialysis treatment for 8 h before the operation was done. The tissue was processed immediately. All laboratory operations were carried out at 0–4°C, unless otherwise stated.

Cell fractionation

Microsomes. After washing off blood and cleaning the cut-up pieces with freshly prepared 0·25 M sucrose, the tissue was homogenized with 7 parts (v/w) of 0·25 M sucrose in a Waring Blendor intermittently for 1 min. The homogenate was centrifuged for 30 min at $12000 \times g_{av.}$ in a Christ Zeta 20 centrifuge. The supernatant was carefully decanted, filtered through four layers of cotton gauze and centrifuged for 15 min at $25000 \times g_{av.}$ using an ultracentrifuge, Beckman model L-2. The resulting supernatant was decanted and centrifuged at $158000 \times g_{av.}$ for 60 min to sediment the microsomes. The microsomal pellets were combined, suspended in 0·25 M sucrose, gently homogenized in a glass-teflon homogenizer and centrifuged for 60 min at $158000 \times g_{av.}$. This procedure was repeated twice. The final microsomal suspension was lyophilized for 18–20 h and stored in suitable portions in glass vials. The vials were kept in a desiccator with Drierite, and the desiccator was placed in a freezer at a temperature of −20°C. Lyophilization of microsomes was found to be necessary, as microsomes stored at −20°C without lyophilization gradually lost activity over a period of two months. The protein content was 8–12 mg/100 mg dry powder. Protein was determined by the method of Lowry *et al.*[8], using dry bovine serum albumin (Serva Entwicklungslabor, Heidelberg, Germany) for the standard curve.

Microsomal subfractionation. Smooth-surfaced and rough-surfaced microsomes were prepared by the method of Dallner *et al.*[9]. A suspension of lyophilized microsomes in 0·25 M sucrose (10–12 mg protein in 3 ml) was layered over 2 ml of 1·3 M sucrose. Both sucrose solutions contained 15 mM CsCl. The centrifugation was carried out in a SW 50 L rotor for 210 min at $130000 \times g_{av.}$. The smooth-surfaced microsomes, which appeared as a whitish band at the interface of the two sucrose layers, were carefully collected with the help of a capillary pipette, provided with a rubber aspirator. The rough-surfaced micro-

somes, which sedimented at the bottom of the centrifuge tube, were gently rinsed with 0·25 M sucrose and suspended by homogenization.

Extraction and chromatography of phospholipids. Total microsomal lipids were extracted and purified by the method of Folch *et al.*[10]. Lyophilized microsomes (100 mg) were homogenized in an all-glass homogenizer with 9·5 ml of chloroform-methanol (2:1, v/v) at 2°C. After addition of 0·5 ml of distilled water, homogenization was continued for another 2 min in an atmosphere of nitrogen.

Thin-layer chromatography plates (DC-Fertigplatten, Kieselgur F_{254}, thickness 2 mm; Merck, Darmstadt, Germany) were washed by the developing solvent, chloroform-methanol–acetic acid–water (25:15:4:2, by vol.). The washing procedure was carried out three times by letting the solvent front reach the top of the plate. The plates were dried and stored in a desiccator with Drierite under vacuum. The plates were activated for 60 min at 110°C before use. Chromatography, detection and elution of phospholipids were carried out as described by Skipski *et al.*[11]. Individual phospholipids from the lyophilized microsomes were further purified by chromatography on silicic acid columns, as described by Ulsamer *et al.*[12]. Phospholipid phosphorus was determined by the method of Chen *et al.*[13].

Preparation of aqueous phospholipid micelles. The purified phospholipids were micellized by sonic oscillation, essentially as described by Jones & Wakil [14]. After removal of the organic solvents the lipid residues were dispersed in 2 ml of ice-cold 0·1 M Tris-HCl buffer, pH 8·0, containing 10 mM EDTA and transferred to a Rosett cooling cell (Branson Instruments, Inc., Danbury, Conn., U.S.A.), designed to hold 2 ml of liquid. The suspension was sonically oscillated at 0°C under an atmosphere of nitrogen for 3 min in a Branson sonifier. The water-clear lipid solution was centrifuged at $43000 \times g_{av.}$ for 60 min to separate solubilized lipids from any nonsolubilized sedimentable material.

Assay of glucuronyltransferase activity. From initial kinetic studies, conditions optimal for enzyme assay were established. These were essentially the same as described previously[1] (time 60 min, temperature 37°C, pH 8·0, 0·05 M Tris-HCl buffer). In addition, the incubation mixture contained 0·01 mM dithiothreitol and 0·1 mM EDTA. Oestriol and UDP-glucuronic acid were present at saturation concentrations; 20–80 μg of enzyme protein was used, and the incubation volume was 1·0 ml. Control incubations did not contain UDP-glucuronic acid. Extraction, measurement of radioactivity and calculation were done as described previously [1].

A unit of enzyme activity was defined as that amount of enzyme necessary to catalyze the formation of 1 nmol of oestriol 16α-monoglucuronide/60 min under the standard assay conditions. Specific activity was defined as units/mg of protein.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was determined by the method of Heppel and Hilmoe[15] in which the release of inorganic phosphate from 5′-AMP is measured.

RESULTS AND DISCUSSION

"Solubilization" of microsomal oestriol 16α-glucuronyltransferase activity. This work is a part of studies in order to look into the possibilities of "solubilizing" and purifying oestriol 16α-glucuronyltransferase as it appears that this enzyme occurs particle-bound. Preliminary studies indicated that deoxycholate released

measurable amounts of enzyme activity from the microsomes into the supernatant. In the present paper, "solubilization" is used as an operational term to define the release of the activity of a particulate enzyme into the supernatant, obtained after centrifugation at $158000 \times g_{av.}$ for 60 min. Treatment of lyophilized microsomes, suspended in buffer of different pH values with deoxycholate (0·05%) indicated that the activity of the "solubilized" enzyme was constant between pH 8·0 and 8·6. It was also observed that the activity released was higher when sucrose was included in the buffer.

In order to find the optimal concentration of deoxycholate which would solubilize the particulate enzyme, lyophilized microsomes were homogenized with 0·25 M sucrose in 0·25 M Tris-HCl buffer, pH 8·0, in an all glass homogenizer. The homogenates were transferred to beakers, and different amounts of a freshly prepared solution of deoxycholate were added. The contents were stirred slowly for 30 min; the mixtures were centrifuged at $158000 \times g_{av.}$ for 60 min. The supernatants were pipetted out, leaving about 1 ml of the clear liquid in the tube over the pellet; concentration of the supernatants was achieved by ultra-filtration using a Diaflo XM-50 filter membrane (cell model 52, Amicon N.V., The Hague, The Netherlands). The apparent discrepancy in the recovery of protein is attributed to non-removal, quantitatively, of the supernatant from the sediment and to aggregation of protein on the XM-50 filter membrane. The ultrafiltrates contained 10–30 μg/ml protein, but were devoid of enzyme activity. The pellets were gently rinsed twice with 0·25 M sucrose in Tris-HCl buffer, pH 8·0, and suspended in the same buffer by homogenization. Table 1 shows the data of the above experiment.

Treatment of lyophilized microsomes with low concentrations of deoxycholate (0·01 and 0·05%) increases the specific activity of oestriol 16α-glucuronyltransferase 2–3 times in the sediment; the specific activity in the supernatant is almost the same as that of the untreated microsomes. It should be noted that microsomal glucuronyltransferase activity is not stimulated in the presence of different concentrations of deoxycholate. With higher concentrations of deoxycholate (0·1 and 0·2%), the activity in the sediment falls abruptly, while that in the supernatant remains the same. The data also show that increasing amounts of deoxycholate led to a corresponding increase in the amount of protein solubilized.

In an effort to increase the yield of oestriol 16α-glucuronyltransferase activity, Mg^{2+}, cysteine, UDP-glucuronic acid and EDTA were included in the medium used to suspend the lyophilized microsomes, each separately and together. In all instances, no increase of enzyme activity in the supernatant was observed. Mg^{2+} alone or in combination with the above mentioned compounds blocked the release of enzymic acitivity, although 40–50% protein was solubilized.

Treatment of the sediments, which possessed high specific activity, with deoxycholate and/or Triton X-100 in the presence and absence of ammonium sulphate (5–50%) led to a considerable decrease in specific activity. Triton X-100, at three different concentrations (0·01, 0·05 and 0·1%), solubilized 45, 55 and 65% protein, respectively, from the lyophilized microsomes; however, the enzymic activity released into the supernatant did not exceed 15%.

Purification of the "solubilized" oestriol 16α-glucuronyltransferase. A suspension of lyophilized microsomes was treated with a solution of deoxycholate (final concentration of deoxycholate in the microsomal homogenate 0·05%). The supernatant obtained after centrifugation at $158000 \times g_{av.}$ for 60 min was concentrated to 1–1·5 ml by ultrafiltration using a Diaflo XM-50 filter membrane.

Table 1. Oestriol 16α-glucuronyltransferase activity in the supernatant fractions and in the sediments after treating lyophilized human liver microsomes with deoxycholate. Deoxycholate was added in different amounts as indicated below; the contents were stirred for 30 min and afterwards centrifuged at 158000 × $g_{av.}$ for 60 min. Lyophilized microsomes were treated with deoxycholate for 30 min and incubated as such

Concentration of deoxycholate[a] (%)	Fraction	Total protein (mg)	Specific activity[b]
0	Microsomes	7·6	44
0·01	Microsomes	7·6	48
	Supernatant	2·0	34
	Sediment	2·0	130
0·05	Microsomes	7·6	50
	Supernatant	3·7	40
	Sediment	1·8	93
0·10	Microsomes	7·6	10
	Supernatant	4·0	39
	Sediment	1·0	21
0·20	Microsomes	7·6	0
	Supernatant	4·5	32
	Sediment	0·75	6
0·50	Microsomes	–[c]	0
	Supernatant	–	0
	Sediment	–	0

[a]The figures in this column represent concentrations of deoxycholate in the microsomal homogenate before centrifugation.
[b]Nanomoles of oestriol 16α-monoglucuronide formed per mg protein per 60 min.
[c]Protein was not determined.

Repeated washing of the residue on the membrane with buffer did not increase the activity of the enzyme. The residue was filtered through a column of Sepharose 4B (1·5 cm × 52 cm). The column was equilibrated with 0·05 M Tris-HCl buffer, pH 8·0 (containing 0·01 mM dithiothreitol and 0·1 mM EDTA); protein was eluted from the column with the same buffer. Fractions of 3 ml were collected. The enzymic activity was found to be present in the ascending shoulder of a protein peak after the void volume (Fig. 1). Fractions having a specific activity greater than that of the microsomes were combined, concentrated to 0·5–1·0 ml by ultrafiltration and passed through another column of Sepharose 4B (1·5 × 50 cm). A single protein peak, with slight trailing emerged. The enzymic activity corresponded with the protein peak. At this stage the specific activity of the "solubilized" enzyme was 5 times higher than that of the lyophilized microsomes (Table 2). Dithiothreitol and EDTA included in the Tris-HCl buffer prevented inactivation of the enzyme, when kept at 2°C for 24 h. Additional efforts to further purify the enzyme, using precipitation with ammonium sulphate, adsorption on DEAE-cellulose, CM-cellulose, DEAE-Sephadex and sucrose density gradient centrifugation did not increase the specific activity of the enzyme.

Properties of the partially purified human liver microsomal oestriol 16α-glucuronyltransferase. Taking into consideration the fact that enzymic activity

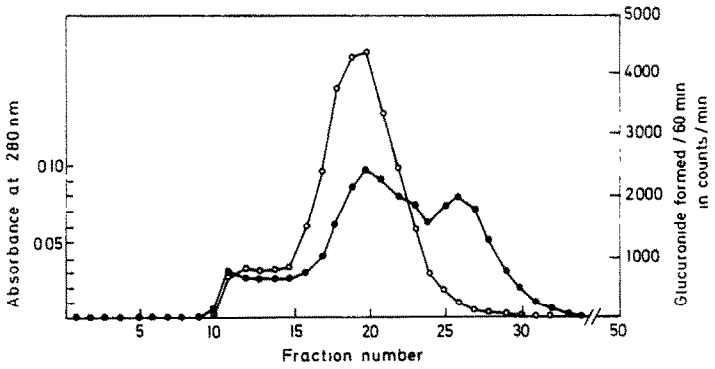

Fig. 1. Elution of a "solubilized" oestriol 16α-glucuronyltransferase of human liver microsomes from a column (1·5 × 52 cm) of Sepharose 4B. The column was eluted with 0·05 M Tris-HCl buffer, pH 8·0, containing 0·01 mM dithiothreitol and 0·1 mM EDTA; 3 ml fractions were collected. Aliquots of 0·5 ml from each fraction were incubated (for details, see "Experimental"); the amount of oestriol 16α-monoglucuronide formed is expressed as radioactive glucuronide formed per 60 min. ●——●. Absorbance of protein at 280 nm; ○——○, radioactive oestriol 16α-monoglucuronide formed per 60 min, in counts/min.

Table 2. Purification of the "solubilized" oestriol 16α-glucuronyltransferase, obtained after treatment of human liver microsomes with 0·05% deoxycholate. The enzyme preparations were incubated with [4-^{14}C] oestriol; conditions of incubation are given in the text

Fraction	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg of protein)
Lyophilized microsomes	15·5	900	100	52
Solubilized enzyme (supernatant)	9·4	540	60	52
XM-50 residue	5·0	400	44	80
First Sepharose 4B-column	0·8	130	14·5	163
Second Sepharose 4B-column	0·07	24	2·7	290

appeared after the void volume, it was thought interesting to compare the ratios of elution volume of the enzyme, of blue dextran and of glutamate dehydrogenase to the void volume of the column. From this result (Table 3) the enzyme appears to have a molecular weight close to 2 million. Centrifugation for 60 min at $130000 \times g_{av.}$ did not sediment enzyme activity. From sucrose density gradient centrifugation the enzyme activity appeared to have a density ranging between 1·059 and 1·07. Analysis on thin-layer plates of silica gel of the phospholipid extract[10] of the purified enzyme showed three minor and two major spots, one of which had the same mobility as that of authentic phosphatidylcholine (Fig. 2). The total microsomal phospholipid extract indicated 8–10 different components.

In order to find out whether this enzyme preparation contained fine structural

Table 3. Ratios of elution volume (V_e) to void volume (V_0) of the partially purified oestriol 16α-glucuronyltransferase from human liver microsomes, blue dextran and glutamate dehydrogenase. Sepharose 4B was equilibrated with 0·05 M Tris-HCl buffer, pH 8·0, containing 0·01 mM dithiothreitol and 0·1 mM EDTA.

Component tested	V_e/V_0
Blue dextran	1·84
Glutamate dehydrogenase	2·1
Oestriol 16α-glucuronyltransferase	1·92
	1·82
	1·84
	1·93[a]
	1·93[b]

[a]Gel filtration was carried out with the incorporation of 0·05% deoxycholate in the buffer.

[b]Gel filtration was carried out with the incorporation of 2 mM dithiothreitol in the buffer.

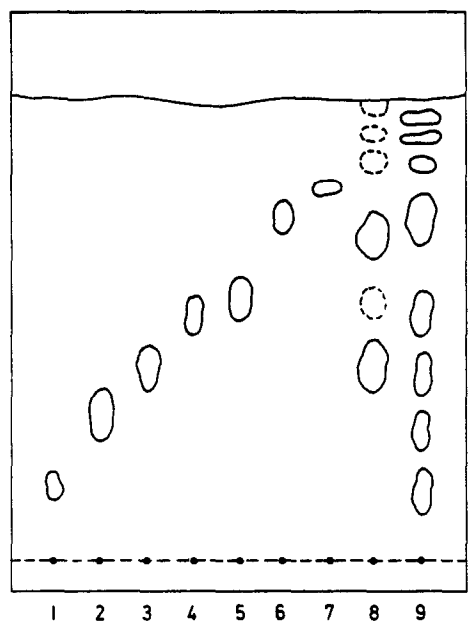

Fig. 2. Thin-layer chromatography of (a) standard phospholipids, (b) phospholipids extracted from the "solubilized" and partially purified oestriol 16α-glucuronyltransferase of human liver microsomes and (c) phospholipids from washed and lyophilized human liver microsomes. The thin-layer plates were developed with chloroform–methanol–acetic acid–water (25:15:4:2, by vol.); the plates were immersed in a jar of iodine vapour to detect the positions of phospholipids. (1) Lysophosphatidyl choline, (2) sphingomyelin, (3) phosphatidyl choline, (4) phosphatidyl inositol, (5) phosphatidyl 1-serine (6) phosphotidyl ethanolamine, (7) cardiolipin, (8) phospholipid extract from the "solubilized" and partially purified enzyme fraction and (9) phospholipid extract from lyophilized human liver microsomes.

fragments from the endoplasmic reticulum, the preparation was fixed for examination under the electron microscope, by mixing with an equal volume of a 2% OsO_4 solution in 0·25 M sucrose, but without deoxycholate, and allowed to stand overnight at 2°C[16]. The mixture was centrifuged in a SW 50 L rotor at $130000 \times g_{av.}$ for 60 min. No sediment could be observed at the bottom of the centrifuge tube. The possibility cannot be excluded that the amount of particles was not sufficient or that the particles are broken down in the presence of OsO_4.

Incubation of the partially purified human liver oestriol 16α-glucuronyltransferase with radioactive estrone and oestradiol-17β resulted in 3·0% glucuronidation of oestrone, while no glucuronidation of oestradiol-17β could be detected. ATP and UDP-*N*-acetylglucosamine did not enhance glucuronidation of oestriol.

The "solubilization" by deoxycholate and subsequent purification of oestriol 16α-glucuronyltransferase from human liver microsomes is characterized by the removal of large amounts of protein with low yield of enzyme activity. Incorporation of different cations, such as Ca^{2+}, Mg^{2+} and Mn^{2+} as well as ATP and UDP-*N*-acetylglucosamine, did not increase the activity. It was thought that the low yield of activity could have been due to the removal of essential factors during the purification procedure. Considering that deoxycholate liberates lipids and proteins from the microsomal membrane, and that some essential phospholipids may have been removed during ultrafiltration or gel filtration, the influence of phospholipids on the partially purified enzyme was studied. Accordingly, different phospholipid components (Fig. 2), in micellized form, from intact microsomes and from the partially purified enzyme were added in increasing amounts to the enzyme preparation (*first* Sepharose 4B-fraction). None of the phospholipids increased the specific activity and thereby the yield of enzyme activity.

The relatively low yield of enzymic activity after the second chromatography on Sepharose 4B was thought to be due to aggregation of lipid and protein components as a result of the removal of deoxycholate during gel filtration. In order to prevent aggregation of lipid and protein, deoxycholate was included throughout the purification procedures. Sepharose 4B columns were equilibrated with 0·05% deoxycholate and filtration of the "solubilized" enzyme was carried out. The elution pattern of protein and enzyme activity did not change to any significant extent, although a slight shift towards the void volume was observed. In the presence of deoxycholate, the enzymic activity was slightly lower than in the absence of deoxycholate. It is likely that deoxycholate liberates lipoprotein subunits of high molecular weight from microsomal membranes and that enzymic activity is associated with these lipoprotein subunits[17]. It appears that the lipoprotein complex, possessing glucuronyltransferase activity, is a unit by itself; separation into different elements may render the enzyme labile.

On filtration through Sepharose 4B enzyme activity was found to have a molecular weight close to two million (The untreated microsomes appeared at the void volume). This high molecular weight of the glucuronyltransferase in addition to the presence of five different phospholipids, is suggestive of the lipoprotein nature of the enzyme.

Treatment of the "solubilized" enzyme with phospholipase C. Incubation of the purified glucuronyltransferase (3 mg) with phospholipase C (0·3 mg) in the presence of 2·5 mM Ca^{2+} at 22°C for 10 min reduced the activity by 70%. After the addition of various phospholipids (lysolecithin, lecithin, sphingomyelin

phosphatidylethanolamine, cardiolipin, phosphatidylinositol and phosphatidyl l-serine) in micellized form (8 and 25 μg) it was found that 25 μg of lysolecithin restored the activity back to 75%. The other phospholipids did not have any significant influence on enzyme activity. It is therefore possible that lipids contribute to the activity of this enzyme. The phospholipid to protein ratio in the purified enzyme preparation was found to be 0·15, while that in the microsomes was 0·32. 5′-Nucleotidase[18] activity could not be detected.

Influence of Mg^{2+} *on enzyme activity.* The specific activity of the microsomal enzyme in the absence of Mg^{2+} was 8·5; however, in the presence of 20 mM Mg^{2+} the activity rose to 52. A similar increase in specific activity was also observed with the "solubilized" and partially purified enzyme preparation. Hence, routinely, incubations were carried out in the presence of 20 mM Mg^{2+}. The oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver[1] was maximally stimulated 50% in the presence of 2·5 mM Mg^{2+}. This difference in stimulatory action of Mg^{2+} on enzyme activity suggests the possibility of different forms of oestriol glucuronyltransferases. In the presence of Mg^{2+} deoxycholate does not liberate enzymic activity. Although the amount of protein "solubilized" remains constant, enzymic activity sediments during centrifugation. It was also observed that the enzyme from the second Sepharose 4B column aggregated when left at 2°C for 20 h in the presence of 20 mM Mg^{2+}. This aggregation was accompanied by an activity loss of 75%; in the absence of Mg^{2+} activity, loss amounted to 25%.

The smooth-surfaced and rough-surfaced microsomes of human liver show differences in glucuronyltransferase activities when oestriol is used as substrate. The smooth-surfaced microsomes were ten times more active than the rough-surfaced microsomes. It is possible that RNP particles on the rough-surfaced microsomes hinder the binding of the substrate to the microsomal membrane; the RNP particles themselves were devoid of enzymic activity.

CONCLUSION

It is clear from the above results that oestriol 16α-glucuronyltransferase "solubilized" and partially purified from the microsomal fraction of human liver is associated with a high molecular weight lipid-protein complex or exists in a highly aggregated state or is tightly bound to particles. The "solubilized" enzyme is stimulated by Mg^{2+}. Phospholipase C inactivates the enzyme, but addition of lysolecithin restores the activity up to 75%. The "solubilized" enzyme is highly specific and, of the three classical oestrogens, conjugates only oestriol. The partially purified enzyme has an apparent molecular weight of approximately two million. This fraction probably contains several protein components of different sizes together with lipids.

ADDENDUM

As this manuscript was being prepared, the work on steroid glycosyltransferases from rabbit tissues appeared. Labow *et al.*[19] also achieved "solubilization" of rabbit liver microsomal glucuronyltransferase by Triton X-100, which conjugated the 17α-hydroxyl group of oestradiol-17α. They were unable to separate the steroid *N*-acetylglucosaminyl transferase from the steroid glucuronyltransferase. The physical properties of the two transferases on filtration through different gel columns led them to the conclusion that the transferases are associated with a particle of very large size. Attempts to break up the active particle by removing lipid destroyed enzymic activity.

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