Effect of Proteases and of Crude Phospholipases on Steroid Glycosyltransferases from Rabbit Liver†

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ABSTRACT: The steroid *N*-acetylglucosaminyltransferase of rabbit liver microsomes was inactivated by a crude phospholipase C which had little effect on the steroid glucuronyltransferase. Protease treatment enhanced the solubilization of the glucuronyltransferase by Triton X-100, but destroyed the *N*-acetylglucosaminyltransferase activity. The glucuronyltransferase was rendered inactive by treatment with snake venom or with 6 M urea, and the activity was partially restored by the addition of crude phospholipid preparations. Chromatography after treatment with 6 M urea showed that partial fragmentation of the particle containing the glucuronyltransferase activity had been achieved. In general, steroid 3-glucosyltransferase activity resembled glucuronyltransferase, while the 17-glucosyltransferase resembled *N*-acetylglucosaminyltransferase in behavior toward the reagents used.

Microsomal preparations of rabbit tissues can effect the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine[1] to the 17-hydroxyl of 17α-estradiol 3-glucuronide, and of glucuronic acid from UDP-glucuronic acid to the phenolic 3-hydroxyl of the steroid estrogens. Labow *et al.* (1971) compared the steroid *N*-acetylglucosaminyl with the steroid glucuronyltransferase activity of Triton-solubilized preparations of rabbit liver and kidney microsomes. They did not achieve a physical separation of the two transferases, but they showed that the enzymes differ greatly in their sensitivity to inhibitors, solubilizing agents, snake venom, and temperature. The present article reports further work on the purification and properties of these two steroid glycosyltransferases, as well as preliminary observations on two UDP-glucose–steroid glucosyltransferases which respectively effect the formation of the 17-glucoside of 17α-estradiol 3-glucuronide (Williamson *et al.*, 1969) and the 3-glucosides of estrone, 17α-estradiol, and 17β-estradiol (Willaimson *et al.*, 1971).

Experimental Section

Materials. Sephadex preparations were obtained from Pharmacia Fine Chemicals, Montreal. Trypsin (type I, recrystallized twice) and α-chymotrypsin (type II, recrystallized three times) were bovine pancreas preparations. These enzymes, as well as phospholipase C (type I, *Clostridium welchii*) and phospholipase D (type I, cabbage) were purchased from Sigma Chemical Co., as were snake venoms, Sepharose preparations, and nucleotides. Urea was reagent grade obtained from Fisher Scientific Co. Soybean lecithin was purchased from Nutritional Biochemicals and synthetic dipalmitoyllecithin and phosphatidyl inositide from General Biochemicals Co. Steroids and steroid conjugates were obtained and purified as described by Jirku and Layne (1965) and Collins *et al.* (1968). Rabbits were mature virgin female New Zealand whites.

Assay of Transferase Activities. Glucuronyltransferase, *N*-acetylglucosaminyltransferase, 3-glucosyltransferase, and 17-glucosyltransferase activities were determined as detailed by Collins *et al.* (1968, 1970) and Labow *et al.* (1971). 17-α-Estradiol-6,7-t_2 and 17α-estradiol-6,7-t_2 3-glucuronoside were used as substrates, respectively, with UDP-glucuronic acid, UDP-*N*-acetylglucosamine, and UDP-glucose as the donor nucleotides.

The protein content of solubilized fractions was measured by the procedure of Lowry *et al.* (1951).

Preparation of Phospholipid Extract from Rabbit Liver Microsomes. The pellet from 10 ml of rabbit liver microsomes, prepared according to Collins *et al.* (1970), was weighed and extracted with chloroform–methanol (2:1). The phospholipid fraction was precipitated from the total lipid fraction by treatment with acetone and 5% magnesium chloride in ethanol according to the procedure of Artom (1957). The phosphate content of each fraction was determined by the method of Fiske and Subbarow as modified by Dittmer and Wells (1969).

Solubilization of Transferase Activity. The solubilization of transferase activities from rabbit liver microsomes with Triton X-100 was carried out as described previously (Collins *et al.*, 1970; Labow *et al.*, 1971). Solubilization with proteases was effected by incubating a suspension of microsomes in 0.15 M Tris-HCl buffer, pH 8.0, for 16 hr at 4° in the presence of 0.03 mg/ml of the protease, either alone or in the presence of 0.1% Triton X-100. After this treatment, the 105,000*g* supernatant was assayed for all four steroid glycosyltransferases.

Snake Venom Treatment. The 105,000*g* supernatant obtained after the treatment of microsomes with trypsin in the presence of Triton X-100 was subjected to treatment with snake venom (*Trimeresurus flavoviridis*), which had previously been heated for 5 min at 100°. The venom was used at a concentration of 0.2 mg/ml in 0.15 M Tris-HCl buffer, pH 8.0, in the presence of 2.5×10^{-3} M calcium chloride. After 2 hr at 4°, the reaction was stopped by the addition of 0.5 ml of 0.15 M EDTA, pH 8.0, and aliquots were assayed for the steroid glycosyltransferases.

Treatment with Phospholipases C and D. The 105,000*g* supernatant, obtained by treatment of microsomes with Triton X-100 in the absence of trypsin, was treated with amounts of phospholipase C ranging from 0.02 to 0.1 mg/ml

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[1] Abbreviations used are: UDP, uridine diphosphate

TABLE I: Effect of Phospholipase Preparations on the Triton X-100 Solubilized Steroid Glycosyltransferases of Rabbit Liver Microsomes.

Treatment of Microsomes	Transferase Activities (pmol of Conjugate Formed per min per mg of Protein)			
	Glucuronide	3-Glucoside	*N*-Acetylglucosaminide	17-Glucoside
Triton X-100	4.76	0.13	1.81	0.34
Triton X-100 followed by				
Trimeresurus flavoviridis venom (0.2 mg/ml)	1.52	0.07	0.93	0.21
phospholipase C (0.07 mg/ml)	4.10	0.10	0.24	0.14
phospholipase D (0.1 mg/ml)	4.52	0.13	1.33	0.31

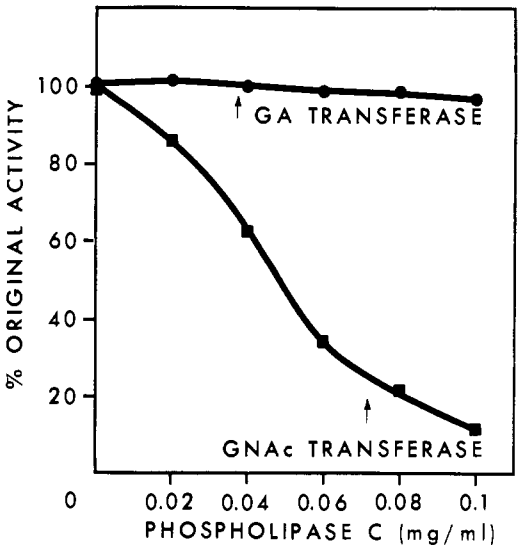

FIGURE 1: Effect of treatment with phospholipase C (*Clostridium welchii*) on the activities of glucuronyltransferase (GA) and *N*-acetylglucosaminyltransferase (GNAc) in Triton-solubilized preparations of rabbit liver microsomes.

in 0.15 M Tris-HCl, pH 8.0, in the presence of 5×10^{-3} M calcium chloride. After 2 hr at 4°, the reaction was stopped with 1 ml of a 0.15 M solution of EDTA, pH 8.0, and aliquots were assayed for the steroid glycosyltransferases. Treatment with phospholipase D was carried out in a similar way.

Chromatography of Glucuronyltransferase Activity. After solubilization of microsomal preparations with trypsin and Triton X-100, 15 ml of the 105,000*g* supernatant was treated with snake venom as described above and was then concentrated to a volume of 3–5 ml by ultrafiltration in an Amicon ultrafiltration cell. Part of this material (1.5 ml) was applied to either a Sephadex G-200, a Sepharose 4B, or a Sepharose 6B column (1.5 × 30 cm) previously equilibrated with 0.15 M Tris-HCl buffer, pH 8.0. The same buffer was used to elute 1.5-ml fractions at a rate of 5–10 ml/hr. The gels were prepared according to manufacturers' specifications and all operations were performed at 4°. Aliquots of the fractions collected were assayed for glucuronyltransferase activity with and without the addition of 20 μl of a solution of rabbit liver phospholipid (1.75 mg of phosphate/ml) in 0.15 M Tris-HCl, pH 8.0. The phospholipid solution was sonicated for 5 min prior to addition to the assay mixture. The protein content of each fraction was estimated by absorbance at 280 mμ in a Cary spectrophotometer.

Chromatography on Sephadex G-200 and Sepharose 6B was also carried out in the presence of 6 M urea. The gels were equilibrated with 6 M urea in 0.15 M Tris, pH 8.0, before and after packing of the column. Cyanate formation was prevented by the addition of 0.1 ml of a 40% solution of methylamine to 100 ml of buffer. The urea buffer was filtered through a Millipore filter before use. One gram of urea was added to 3.0 ml of the concentrated preparation obtained by digestion of microsomes with trypsin in Triton X-100, followed by snake venom treatment. This brought the enzyme solution to a concentration of 6 M with respect to urea, and 1.5 ml of this solution was applied to the column. The procedure for elution and assay of the fractions was the same as that described for the column without urea.

Results

Effect of Phospholipase C and D on Transferase Activities. Table I shows the effect of three phospholipase preparations on the transferase activities in the supernatant obtained by treatment of rabbit liver microsomes with Triton X-100. In accord with previous results (Labow *et al.*, 1971) the *N*-acetylglucosaminyltransferase was not inactivated to the same extent as the glucuronyltransfease by treatment with snake venom. In contrast, phospholipase C strongly inactivated *N*-acetylglucosaminyltransferase at concentrations which had little effect on glucuronyltransferase (Figure 1). Attempts to restore the activity of *N*-acetylglucosaminyltransferase by the addition of phospholipid preparations gave variable results but the extent of the restoration was never greater than 15–25% of the original activity. The 3-glucosyltransferase resembled glucuronyltransferase in its sensitivity to snake venom and phospholipase C, while the

TABLE II: Comparison of the Effect of Trypsin and of Chymotrypsin, Alone or in Combination with Triton X-100, on the Solubilization of Steroid Glycosyltransferases from Rabbit Liver Microsomes.

Treatment of Microsomes	Transferase Activities (pmol of Conjugate Formed per min per mg of Protein)			
	Glucuronide	3-Glucoside	*N*-Acetylglucosaminide	17-Glucoside
Trixon X-100	6.10	1.60	1.81	0.34
Trypsin	5.00	0.98	0.00	0.00
Trypsin + Triton X-100	7.30	1.50	0.00	0.00
Chymotrypsin	3.40	1.15	0.00	0.00
Chymotrypsin + Triton X-100	7.29		0.00	0.00

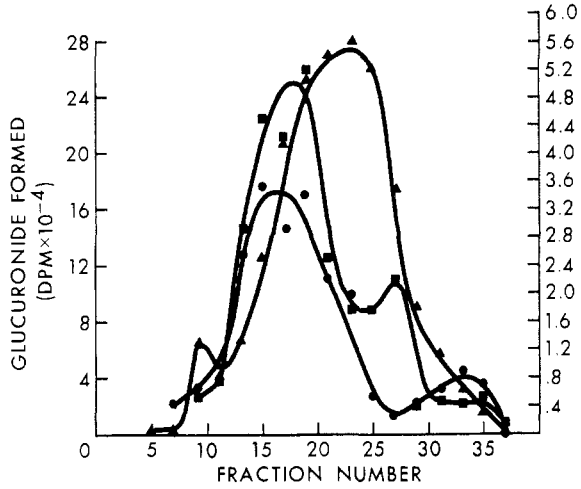

FIGURE 2: Elution pattern on Sepharose 6B of steroid glucuronyltransferase in rabbit liver microsomes treated successively with trypsin in Triton X-100 and with *Trimeresurus flavoviridis* venom: (▲) protein measured as OD at 280 mμ; enzyme activity before (●) and after (■) the addition of rabbit liver phospholipid to the column fractions.

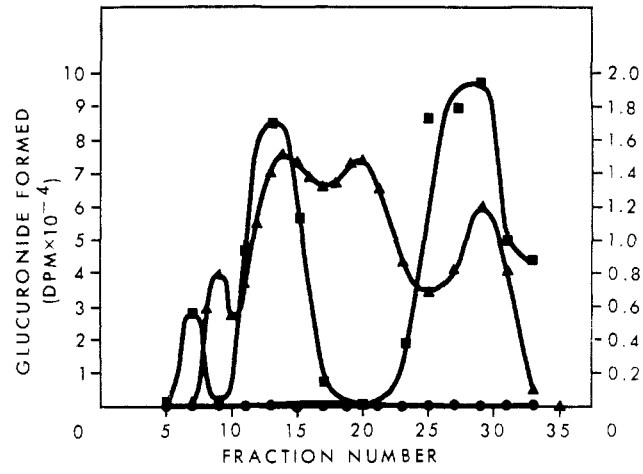

FIGURE 3: Elution pattern on Sepharose 6B of steroid glucuronyltransferase in rabbit liver microsomes treated successively with trypsin in Triton X-100, *Trimeresurus flavoviridis* venom, and 6 M urea: (▲) protein measured as OD at 280 mμ; enzyme activity before (●) and after (■) the addition of rabbit liver phospholipid to the column fractions.

17-glucosyltransferase was essentially similar to *N*-acetylglucosaminyltransferase in its behavior in these experiments.

Phospholipase D had little effect on the activity of any of the steroid glycosyltransferases under the standard assay conditions (Table I). Attempts to perform experiments at the optimum pH of phospholipase D, namely 5.6, were unsuccessful, since all the transferases were inactive at this pH.

Effect of Proteases on Transferase Activities. Table II shows the effect of treatment with trypsin and with chymotrypsin on the solubilization by Triton X-100 of the individual steroid glycosyltransferases. All four transferases were partially solubilized by Triton X-100 as previously found by Labow *et al.* (1971). When either trypsin or chymotrypsin was included with Triton X-100 in the solubilization mixture, both *N*-acetylglucosaminyltransferase and 17-glucosyltransferase were completely inactivated in both the 105,000*g* supernatant and the pellet. The total glucuronyltransferase activity released into the 105,000*g* supernatant by either trypsin or chymotrypsin was enhanced by a factor of 2–3. Protease VI, protease VII, and papain had similar effects on the transferase activities.

TABLE III: Reduction by Snake Venom of the Steroid Glucuronyltransferase Activity of a Triton X-100 Trypsin Treated Preparation of Rabbit Liver Microsomes, and the Restoration of the Transferase Activity by Addition of Various Phospholipid Preparations.

	Glucuronyltransferase Act. (pmol per min per mg of Protein)
Triton X-100 trypsin digest	
before snake venom treatment	2.60
after snake venom treatment	1.02
Phospholipid added after venom treatment	
total lipid fraction from rabbit liver (39 (μM P_i)	1.53[a]
phospholipid fraction from rabbit liver (0.22 μM P_i)	1.52
soybean lecithin (300 μg)	1.76
phosphatidylethanolamine (300 μg)	1.15
phosphatidylinositol (300 μg)	1.33
cardiolipin (150 μg)	1.44
dipalmitoyllecithin (300 μg)	1.32

[a] Each phospholipid tested at a variety of concentrations. The values given are those for the concentration at which the best reactivation was obtained.

Reconstitution of Glucuronyltransferase Activity after Treatment with Snake Venom. Table III shows the decrease of glucuronyltransferase activity, caused by treatment with snake venom, in the microsomal preparation obtained by treatment with trypsin in the presence of Triton X-100. The activity was partially restored by the addition of phospholipid to the snake venom treated material. As described previously (Labow *et al.*, 1971) the extent of the inactivation by snake venom was about 75%. The extent of the restoration of activity varied from one phospholipid preparation to another, and the addition of excessive amounts of phospholipid resulted in further inhibition rather than restoration of the glucuronyltransferase activity.

Chromatography of Snake Venom Microsomal Extract. Practically all of the glucuronyltransferase activity in the material obtained by treatment with snake venom of a liver microsomal preparation solubilized with trypsin in the presence of Triton X-100 was eluted with the void volume from a column of Sephadex G-200 (exclusion limit mol wt, 8×10^5). When chromatography was carried out on Sepharose 6B (exclusion limit mol wt, 4×10^6) the activity was retained on the column (Figure 2). Treatment of the microsomal extract with 6 M urea prior to chromatography resulted in almost total loss of glucuronyltransferase activity, which was restored to the extent of about 50% by the addition of phospholipid. Preliminary chromatography on Sephadex G-200 indicated that part of this restored glucuronyltransferase activity was retained on the column to a significant extent.

Chromatography on Sepharose 6B in 6 M urea produced two discrete major peaks of activity as shown in Figure 3.

Discussion

The previous results of Labow *et al.* (1971) indicated that the UDP-glucuronic acid–steroid glucuronyltransferase and the UDP-*N*-acetylglucosamine–steroid *N*-acetylglucosaminyltransferase in rabbit liver were, even after "solubilization" with Triton X-100, still contained in particles of very large size. In the present work, treatment of the microsomes with trypsin and other proteases increased the amount of glucuronyltransferase activity in the Triton X-100 extract (Table II). However, even after treatment of this material with *Trimeresurus flavoviridis* venom, the residual glucuronyltransferase activity was still eluted with the void volume on Sephadex G-200. The results of treatment of the microsomal extract with 6 M urea prior to chromatography are of considerable interest. The total loss of glucuronyltransferase activity by this treatment and its partial restoration by the addition of phospholipid suggest that the 6 M urea destroys the structural feature of the microsomal membrane on which glucuronyltransferase activity may depend (Graham and Wood, 1969). The action of 6 M urea, in contrast to that of snake venom, resulted in a considerable fragmentation of the particle containing the glucuronyltransferase, and at least two discrete peaks of enzyme activity were obtained by chromatography of the resulting material on Sepharose 6B after partial reconstitution with phospholipid (Figure 3). This results in a partially purified preparation of steroid glucuronyltransferase which is devoid of *N*-acetylglucosaminyl- and glucosyltransferase activities, since these do not survive the treatment with proteases (Table II).

The fact that phospholipase C markedly reduces the activity of *N*-acetylglucosaminyltransferase at concentrations which have little effect on glucuronyltransferase activity (Figure 1) is in contrast to the fact that the latter enzyme is the more sensitive to *Trimeresurus flavoviridis* venom, as shown by Labow *et al.* (1971) and confirmed in the present work (Table II). This suggests that there is a different arrangement of polar head groups in the phospholipid matrix required for the activity of these transferases. The relative insensitivity of the glucuronyltransferase to proteases, as compared to both the glucosyl- and the *N*-acetylglucosaminyltransferases, might mean that the latter enzymes require a much greater degree of structural integrity of the microsomal membrane for activity.

The phospholipid preparations used in this work to effect reconstitution of transferase activity were all relatively crude mixtures. Similarly, the phospholipase preparations were not highly purified with regard to their specified activity. It therefore is not possible to use the present results as a basis for speculation as to the precise structural characteristics required for the activity of either the glucuronyl- or the *N*-acetylglucosaminyltransferase.

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