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Properties of Some Steroid Glycosyl Transferases from Rabbit Tissues*

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ABSTRACT: The steroid *N*-acetylglucosaminyl and glucuronyl transferases in Triton-solubilized preparations of rabbit liver and kidney microsomes were not effectively separated by chromatography on a variety of ion-exchange and gel filtration columns or by centrifugation or electrophoresis in sucrose density gradients. The *N*-acetylglucosaminyl transferase was, however, selectively inhibited by UTP and by thiol reagents, and was not, in contrast to the glucuronyl transferase, solubilized by cetyltrimethylammonium bromide. The *N*-acetylglucosaminyl transferase was more stable than the glucuronyl transferase to treatment with snake venom and to heating at 55°. Selective use of these procedures, together with chromatography on Sepharose 2B, can be used to obtain preparations of either transferase essentially free of the other.

There is now a great deal of evidence that the UDP-glucuronic acid:steroid glucuronyl transferase in animal tissues consists of a group of enzymes with similar physical properties and different substrate specificities (Rao and Breuer, 1969). Two UDP-*N*-acetylglucosamine:steroid *N*-acetylglucosaminyl transferases have been investigated. That described by Collins *et al.* (1968) in the rabbit has a strict specificity for the 17α-hydroxyl group of phenolic steroids which carry an acidic conjugating group on the 3 position. This enzyme therefore differs from the transferase described by Levitz and his collaborators (Cable *et al.*, 1970) in the human, which seems to be specific for the 15α-hydroxyl group on phenolic steroids, and will transfer *N*-acetylglucosamine to this group in the presence or absence of a sulfate radical at the 3 position of the steroid. The physiological role of these steroid *N*-acetylglucosaminyl transferases is unknown, and this work was undertaken to investigate means for their purification and further study.

Experimental Section

Materials. Sephadex preparations were obtained from Pharmacia Fine Chemicals, Montreal. Snake venoms, CM-cellulose, DEAE-cellulose, Sepharose preparations, and nucleotides were purchased from Sigma Chemical Co. Steroids and steroid conjugates were obtained and purified as described by Jirku and Layne (1965) and Collins *et al.* (1968). Calcium phosphate gel was prepared according to the procedure of Swingle and Tiselius (1951). Rabbits were mature virgin female New Zealand whites.

Assay of Transferase Activities. Glucuronyl transferase and *N*-acetylglucosaminyl transferase activities were determined as detailed by Collins *et al.* (1968, 1970) using 17α-estradiol-*6,7-t* and 17α-estradiol-*6,7-t* 3-glucuronide as substrates, respectively, with UDP-glucuronic acid and UDP-*N*-acetylglucosamine as the donor nucleotides. The only change from

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TABLE I: Effect of Solubilization Techniques on Activity of Glucuronyl and *N*-Acetylglucosaminyl Transferases of Rabbit Liver Microsomes.

	pmoles of Conjugate Formed per min per mg of Protein			
	Supernatant 105,000*g*		Microsomal Pellet	
Treatment	Glucuronide	*N*-Acetylglucosaminide	Glucuronide	*N*-Acetylglucosaminide
Triton X-100	12.8	6.7	20.8	4.2
Cetyltrimethylammonium bromide	24.9	1.2	14.4	0.9
Sonication 2 min	12.8	2.8	8.7	2.3
Sonication 5 min	15.5	2.9	5.9	3.5

the published assay procedure was the inclusion of 0.03 mM $MnCl_2$ in the buffer. The protein content of tissue fractions was measured by the procedure of Lowry *et al.* (1951).

Solubilization and Fractionation of Transferase Activity. The solubilization of transferase activities from rabbit liver and kidney microsomes with Triton X-100, and fractionation of the solubilized material with ammonium sulfate, were carried out as described previously (Collins *et al.*, 1970). The amount of ammonium sulfate to be added was calculated from the chart provided by Di Jeso (1968). Solubilization with other detergents was effected in a similar manner using the following concentrations in 0.15 M Tris-HCl buffer–cetyltrimethylammonium bromide (0.3%), sodium deoxycholate (0.05%), and digitonin (1.0%). Sonication was done with a Bronwill probe-type ultrasonicator.

Snake Venom Treatment. Separate 1.0-ml portions of the Triton X-100 supernatant prepared from liver and kidney microsomes were treated with increasing amounts of snake venom (*Trimeresurus flavoviridis*) at 4° for 2 hr. Aliquots were removed and assayed for the steroid glycosyl transferases.

Chromatography of Transferase Activities. After solubilization with Triton X-100, the precipitate obtained by treatment of the solubilized material with 35–60% ammonium sulfate was resuspended in 1–3 ml of 0.01 M Tris-HCl buffer at pH 8.0, and the solution was desalted on a 1 × 15 cm column of Sephadex G-15. The eluate was applied to columns of various ion-exchange resins and of calcium phosphate gel, and eluted with appropriate buffers. Resin columns were prepared according to the manufacturers' specifications and all chromatographic operations were performed in a cold room at 4°. For chromatography on gel filtration columns, the 35–60% ammonium sulfate fraction was resuspended in 1–3 ml of 0.15 M Tris-HCl (pH 8.0) and was not desalted prior to application to the columns, which were preequilibrated in all cases with the same buffer.

Sucrose Density Gradient Centrifugation. Aliquots of the Triton X-100 supernatant and of sonicated microsomes from liver were each applied in 0.6 ml of 0.15 M Tris-HCl (pH 8.0), to a sucrose density gradient with a volume of 3.0 ml and concentration of sucrose ranging from 5 to 20%. The gradients were centrifuged at 56,000 rpm in an SW56 Beckman rotor for 2.5 hr. The tubes were punctured and 10 drops of each fraction were collected. Sonicated microsomes (0.6 ml in 0.15 M Tris-HCl, pH 8.0) were layered on 3.0 ml of a sucrose density gradient ranging in concentration from 10 to 40% sucrose. The tubes were centrifuged for 3 hr at 47,500 rpm in a SW56 rotor, and were then punctured to permit the collection of 8 drops/fraction.

Sucrose Density Gradient Electrophoresis. The liver microsomal suspension (10 ml) was diluted to 50 ml with 0.01 M Tris-HCl buffer at pH 8.8. A Triton X-100 supernatant was prepared as described by Collins *et al.* (1970). The 35–60% ammonium sulfate fraction was desalted on a Sephadex G-10 column and 5.0 ml of the eluate was applied to a sucrose density gradient electrophoresis column (LKB, 3340) with a volume of 100 ml, ranging in concentration from 20 to 50% sucrose in 0.01 M Tris-HCl (pH 8.8) (Svensson, 1960). A potential difference of 1000 V was applied across the gradient for 2.5 hr. At the end of this period, the electrophoresis was stopped and 2.5-ml fractions were collected and assayed for glucuronyl transferase and *N*-acetylglucosaminyl transferase activities.

Results

Solubilization of Transferases in Liver and Kidney. The kidney enzymes are solubilized by Triton X-100 in the same manner as has previously been reported for the liver enzymes (Collins *et al.*, 1970). The specific activity of the *N*-acetylglucosaminyl transferase in the kidney is lower than that in liver, but the kidney exhibits a higher *N*-acetylglucosaminyl transferase than glucuronyl transferase activity. The glucuronyl transferase activity of kidney tissue was so low as to approach the lower limit of sensitivity of the assay method, and it was not studied further in the experiments recorded below.

Treatment of liver microsomes with sodium deoxycholate and with digitonin effected considerable solubilization of both transferase activities as measured by their retention in the supernatant after centrifugation at 105,000*g* for 1 hr. However, these reagents were less effective than Triton X-100, and they both showed some inhibition of the transferases at the concentrations needed to effect solubilization. Cetyltrimethylammonium bromide at a concentration of 0.3% was a good agent for the solubilization of glucuronyl transferase, but caused almost complete destruction of the *N*-acetylglucosaminyl transferase. Sonication solubilized both enzyme activities, but was somewhat more effective on glucuronyl transferase. Table I compares the effect of Triton X-100, cetyltrimethylammonium bromide, and sonication on the distribution of the liver microsomal transferases in the soluble and sedimentable fraction after centrifugation at 105,000*g*.

Characteristics of Liver and Kidney Transferases. The pH–activity relationship of kidney *N*-acetylglucosaminyl transferase was determined in phosphate buffer using the Triton X-100 supernatant. The optimum was found to be 7.8 and the shape of the curve was similar to that found by Collins *et al.* (1968) for the *N*-acetylglucosaminyl transferase from rabbit liver microsomes.

The steroid glucuronyl transferase of liver is inhibited by a wide variety of alcohols and steroids which serve as substrates for the transferase (Collins *et al.*, 1968). In Table II the inhibitory effect of several compunds on glucuronyl transferase and *N*-acetylglucosaminyl transferase is compared. The sulfhydryl reagents *p*-chloromercuribenzoate and dithiobisdinitrobenzoic acid were potent inhibitors of *N*-acetylglucosaminyl transferase, but had no effect on glucuronyl transferase at a

TABLE II: Effect of Various Compounds on the Activity of Triton-Solubilized Glucuronyl (GA) Transferase and *N*-Acetylglucosaminyl (GNAc) Transferase of Rabbit Tissues.

Compound Added to Incubation Medium	Concn (M)	% Inhibition		
		Liver GA Transferase	Liver GNAc Transferase	Kidney GNAc Transferase
Phenolphthalein	1×10^{-4}	22	4	55
Eugenol	2.5×10^{-4}	9	0	9
Estrone	4.0×10^{-5}	51	0	0
17α-Estradiol	4.0×10^{-5}	70	19	39
Diethylstilbestrol	4.0×10^{-5}	40	13	24
Uridine triphosphate	3×10^{-5}	10	43	56
p-Chloromercuribenzoate	1×10^{-6}	<5	89	70
Dithiobisdinitrobenzoic acid	1×10^{-6}	<5	77	64

concentration of 10^{-6} M. The K_I for diethylstilbestrol was determined for both transferases by the method of Dixon and Webb (1964). The inhibition is competitive in both cases. Neither ATP nor *N*-acetylglucosamine inhibited either transferase, but UTP was a potent inhibitor of *N*-acetylglucosaminyl transferase. This inhibition was noncompetitive.

Stability and Temperature Sensitivity of Transferases. The transferase activities in liver microsomal preparations were reduced by about 50% by storage at −10° for 1 month. The kidney microsomal preparation contained no detectable transferase activity after similar storage. When the microsomal preparations were lyophilized prior to storage, the transferase activities in both tissues were stable for up to 3 months. Triton X-100 supernatants from both liver and kidney lost 50% of the transferase activities when left overnight at 4°, and 90% when left overnight at room temperature. No major difference was detected between the stability of the glucuronyl transferase and the *N*-acetylglucosaminyl transferase activities to these treatments. However, as shown in Figure 1, the glucuronyl transferase was more rapidly inactivated by heating to 55–60° than was the *N*-acetylglucosaminyl transferase.

Effect of Snake Venom. The results in Figure 2 show that glucuronyl transferase was much more sensitive to the venom of *T. flavoviridis* than was the *N*-acetylglucosaminyl transferase. The latter activity was inhibited to the extent of only 15% by a concentration of venom which provided more than 80% destruction of glucuronyl transferase.

Chromatography of Transferases. Both transferase activities in the Triton-solubilized fraction of microsomes were retained on columns of DEAE-Sephadex, QAE-Sephadex, and DEAE-cellulose, from which they were not eluted by buffers of high ionic strength. They were eluted from these resins by a solution of 0.05% Triton X-100 in 0.5 M Tris-HCl (pH 8.0). Partial inactivation of the enzymes occurred, with altered ratios of glucuronyl transferase to *N*-acetylglucosaminyl transferase

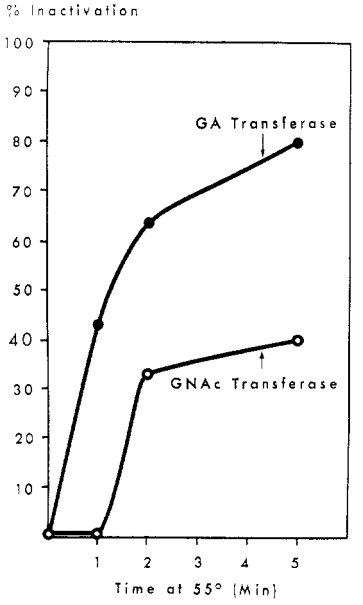

FIGURE 1: Effect of heat on glucuronyl (GA) transferase and *N*-acetylglucosaminyl (GNAc) transferase activities in Triton-solubilized preparations of rabbit liver microsomes.

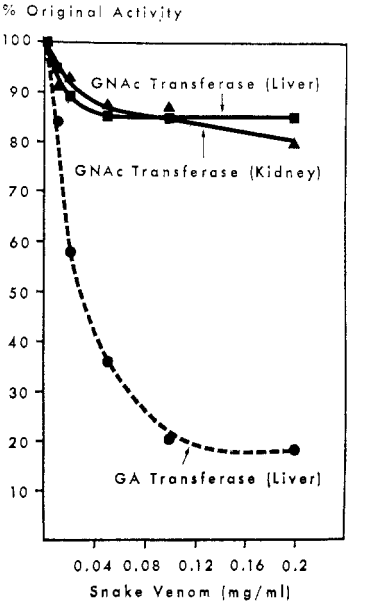

FIGURE 2: Effect of treatment with snake venom (*Trimeresurus flavoviridis*) on the activities of glucuronyl (GA) transferase and *N*-acetylglucosaminyl (GNAc) transferase in Triton-solubilized preparations of rabbit liver microsomes.

TABLE III: Effect of Addition of Manganese Chloride to the Incubation Medium on the Activity of Steroid Glucuronyl (GA) Transferase and *N*-Acetylglucosaminyl (GNAc) Transferase of Rabbit Liver.

	pmoles of Conjugate Formed per min per mg of Protein			
	GA Transferase		GNAc Transferase	
Concentration (mM) $MnCl_2$ added	0	0.03	0	0.03
Fraction assayed				
Microsomes	13.2	13.2	2.5	2.7
Triton X-100 supernatant	14.2	14.4	1.6	3.5
$(NH_4)_2SO_4$ 35–60%	18.2	20.1	5.3	6.3
Sepharose 2B peak	22.3	25.7	4.8	7.1

activities in the eluates as compared to the material applied to the columns, but separation of the two transferase activities was not achieved.

The transferases were not adsorbed on either CM-Sephadex or CM-cellulose. They both adhered strongly to calcium phosphate gel, and were only partially eluted by 1.0 M phosphate buffer at pH 6.0. There was considerable loss of specific activity and alteration of the ratios of glucuronyl transferase to *N*-acetylglucosaminyl transferase, but no separation of the two activities was observed. Both the *N*-acetylglucosaminyl transferase and the glucuronyl transferase activities were retained on Sepharose 2B, 4B, and 6B, which have exclusion limits of mol wt 2×10^7, 8×10^6, and 4×10^6, respectively. They were eluted together in the void volume from columns of Sephadex G-200 (exclusion limit mol wt 8×10^5). None of these column procedures yielded preparations with major increase of specific activity over that of whole microsomes. The best purification was obtained with Sepharose 2B, which gave a threefold purification of both transferases in terms of protein, provided that the column eluates were assayed in the presence of $MnCl_2$ as described below (Table III).

Requirement for Manganese. When the Triton-solubilized transferase preparations were dialyzed overnight against 0.01 M Tris-HCl (pH 8.0) the activity of the *N*-acetylglucosaminyl transferase was completely lost, while that of the glucuronyl transferase was reduced by about 10–15%. Both activities were restored by the addition of $MnCl_2$ (Bosmann, 1970) to the assay medium at a concentration of 0.03 mM. This led to the routine inclusion of this concentration of $MnCl_2$ for assays of column fractions and other purified transferase preparations (Table III).

The resin and gel chromatographic procedures were repeated on separate fractions of the Triton-solubilized fraction of liver microsomes which had been subjected to the following treatments, sonication, dialysis against EDTA, followed by homogenization with deoxycholate (Halac and Reff, 1967), digitonin (Winsnes, 1969), exposure to snake venom, lysosomal digestion (Takesue and Omura, 1969), and a variety of organic solvents. None of these treatments reduced the size of the active particle containing the transferases nor improved the separation of the two activities by the chromatographic procedures.

Sucrose Density Gradient Centrifugation and Electrophoresis. The Triton X-100 supernatant was dispersed throughout the entire gradient when centrifuged in a sucrose density gradient. In contrast, the sonicated microsomes sedimented to the bottom of the tubes. Both transferases were found in the same fractions of the gradient. On electrophoresis, two protein bands were obtained, which migrated, respectively, 3.0 and 5.0 cm toward the anode. Both transferase activities were located in the slower moving band.

Discussion

The results indicate that the UDP-*N*-acetylglucosamine: steroid *N*-acetylglucosaminyl transferase present in rabbit liver microsomes is very similar in physical properties to the UDP-glucuronic acid:steroid glucuronyl transferase which forms the 3-glucuronides of the phenolic estrogens. Both these glycosyl transferases also occur in rabbit kidney microsomes, and no difference in physical properties was detected between the *N*-acetylglucosaminyl transferases in liver and kidney. The level of the glucuronyl transferase in kidney was very low and detailed investigation of this enzyme was not carried out.

Treatment with Triton X-100 was the method of choice for obtaining a soluble preparation of both glucuronyl and *N*-acetylglucosaminyl transferase (Table I). However, cetyltrimethylammonium bromide is a useful reagent in that it provides a preparation of glucuronyl transferase almost devoid of *N*-acetylglucosaminyl transferase activity. Conversely, the latter activity can be obtained with a much reduced glucuronyl transferase content by heating the Triton-solubilized microsomal preparation at 55° for 1 min (Figure 1) or by treatment of the preparation with snake venom (Figure 2). These differences in the effects of various treatments on the two enzyme activities reinforce the evidence obtained by Collins *et al.* (1968) from tissue distribution studies, which suggested that the *N*-acetylglucosaminyl transferase was an enzyme physically distinct from the glucuronyl transferase. This conclusion is supported by the different behavior of the transferases toward the inhibitors shown in Table II. Of particular interest is the inhibition of *N*-acetylglucosaminyl transferase by the sulfhydryl reagents *p*-chloromercuribenzoate and dithiobisdinitrobenzoic acid, as well as by UTP, at concentrations which have little effect on glucuronyl transferase. Inhibition by UTP is a characteristic of other glycosyl transferases which employ UDP-*N*-acetylglucosamine as the donor nucleotide (Tuppy and Schenkel-Brunner, 1969).

Collins *et al.* (1968) described the inhibition of rabbit liver steroid *N*-acetylglucosaminyl transferase by diethylstilbestrol, although no *in vitro* formation of an *N*-acetylglucosaminide of this compound could be detected. These results were confirmed in the present experiments, and in an unpublished experiment in this laboratory a careful search failed to detect radioactive *N*-acetylglucosaminide conjugates in the urine of a rabbit to which [^{14}C]diethylstilbestrol had been administered intravenously. Nonetheless, the inhibition by diethylstilbestrol of the transfer of *N*-acetylglucosamine to 17α-estradiol 3-glucuronide follows the kinetics characteristic of competitive inhibition (Dixon and Webb, 1964) and it seems probable, therefore, that diethylstilbestrol must inhibit the steroid *N*-acetylglucosaminyl transferase by binding to the enzyme without proceeding to the formation of product.

The results obtained on gel filtration suggest that the transferase activities after solubilization with Triton X-100 are still contained in a particle of very large size. The broad bands obtained on sucrose density gradient centrifugation, and the several broad peaks obtained by filtration on Sepharose 2B

4B, or 6B both suggest the presence of active particles of several molecular sizes. All attempts to break up the active particle by removing lipid resulted in complete destruction of the enzyme activities, which were not restored by the addition to the lipid-free extracts of micellar acetone extracts of microsomes.

At present, complete separation of the steroid glucuronyl and *N*-acetylglucosaminyl transferases with retention of both activities has not been achieved. However, the transferases can be selectively inhibited or destroyed in the solubilized microsomal extracts, and these procedures, together with filtration on Sepharose 2B, can be used to obtain partially purified preparations of either transferase activity essentially devoid of the other.

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Control of Estrogen Binding Protein Concentration under Basal Conditions and after Estrogen Administration*

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ABSTRACT: The control of estrogen receptor concentration in the immature rat uterus was studied under basal conditions (without estrogen), and after estrogen treatment. An estimate of the turnover rate of the cytoplasmic estrogen receptor was determined using cycloheximide to block protein synthesis. At various times after exposure to the inhibitor, the estrogen binding was assayed. When protein synthesis had been blocked 8–12 hr either *in vivo* or *in vitro*, the total binding capacity decreased only 6%. The turnover rate of the binding protein (estimated half-life of 5–6 days) was much slower than the half-life of 20–22 hr previously reported for the proteins of uterine cytosol. After a single intraperitoneal injection of 0.1 μg of 17β-estradiol, the estrogen binding capacity of the cytoplasmic estrogen receptor decreases by 50%. The binding capacity is replenished beginning about 6 hr after the estrogen injection, and by 16 hr the estrogen receptor level reaches control values. Cycloheximide or actinomycin D, when administered shortly before or 2 hr after estrogen, inhibits the return of estrogen binding in the cytosol. Cycloehximide or actinomycin D administered *in vivo* 6 hr after estrogen has little or no effect on the level of estrogen receptor binding. The data indicate that binding capacity is replenished at a time when synthesis of the binding protein does not occur. It also appears that both protein synthesis and RNA synthesis are involved in an early event that is essential for the replenishment of the estrogen binding protein seen after an *in vivo* surge of estrogen.

When 17β-estradiol is injected into immature or ovariectomized rats, it is selectively taken up and bound in tissues which are responsive to estrogens, such as the uterus, vagina, mammary glands, and pituitary gland. This fact has led to intensive investigations concerning the entity responsible for this binding, the so-called estrogen receptor (Jensen and Jacobson, 1962; Noteboom and Gorski, 1965; Toft and Gorski, 1966; Shyamala and Gorski, 1969). The 105,000*g* supernatant fraction (cytosol) of uterine cells contains a factor which specifically binds estrogens, *in vivo* or *in vitro*, and which sediments in a linear sucrose density gradient at about 8 S (Toft *et al.*, 1967; Erdos, 1968). Studies of this receptor's binding specificity, its size (mol wt ~200,000), and its sensitivity to proteolytic enzymes suggest that it is a protein (Noteboom and Gorski, 1965; Toft and Gorski, 1966).

The estrogen "receptor" concentration in the cytosol remains fairly constant at a calculated value of 16,000 binding sites/cell in the immature rat (Clark and Gorski, 1970) and in the ovariectomized rat (Notides, 1970). After estrogen injection, the cytosol receptor numbers fall as the estrogen–receptor complex apparently moves into the nucleus. After several

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