Biochemical Pharmacology, Vol. 24, pp. 655–662. Pergamon Press, 1975. Printed in Great Britain.

STUDIES ON BILIRUBIN AND STEROID GLUCURONIDATION BY RAT LIVER MICROSOMES

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(*Received* 26 *June* 1974; *accepted* 23 *August* 1974)

Abstract—The addition of digitonin or other detergents to liver microsomes from control and phenobarbital-treated rats increased the rate of glucuronidation of bilirubin, testosterone, estradiol, estrone and estriol. The extent of activation by digitonin as well as the concentration of detergent causing maximal activation was substrate dependent. Treatment of rats with sodium phenobarbital (75 mg/kg/day, i.p.) for 3 days significantly increased the glucuronidation of testosterone and estradiol per mg of protein but had no effect on estrone glucuronidation. Administration of 0·1% sodium phenobarbital in the drinking water for 20 days increased bilirubin, testosterone and estrone glucuronidation per mg of protein, but had little or no effect on estradiol and estriol glucuronidation. When UDP-glucuronyl transferase activity was calculated on a per g liver (wet wt) basis, however, an increase in the glucuronidation of estradiol and estriol was seen; this increase could be accounted for by a large stimulatory effect of phenobarbital on the synthesis of total microsomal protein. Liver microsomes from homozygous Gunn rats, which are deficient in the ability to conjugate bilirubin, did conjugate testosterone, estradiol, estrone and estriol, but some substrates were conjugated more rapidly than others. The addition of digitonin to liver microsomes from the homozygous Gunn rat increased the glucuronidation of testosterone but not that of estrone. Microsomal steroid glucuronidation in this animal was stimulated to varying degrees by the administration of sodium phenobarbital. The data suggest the presence of several glucuronyl transferases in liver microsomes.

Many endogenous and exogenous compounds are metabolized in the body by a conjugation reaction involving hepatic microsomal UDP-glucuronyl transferase. This enzyme catalyzes the transfer of glucuronic acid from UDP glucuronic acid (UDPGA) to the aglycone, which may be an alcoholic, phenolic, carboxylic or amino compound, with the resultant formation of the *O*- or *N*-glucuronide.

There is considerable evidence which suggests that a multiplicity of glucuronyl transferases exist in liver microsomes. This evidence is based on the ability of the homozygous Gunn rat and the cat to conjugate certain substances and not others [1–3], the separation of some glucuronyl transferase activities from each other during the course of purification [4, 5], the absence of mutual inhibition of glucuronidation between various substrates [6–10] as well as the absence of mutual product inhibition [11], differences in the response of the rate of conjugation to metals and other compounds [5, 11–14], differences in submicrosomal distribution of activity for several substrates [15, 16], and differences in the rates of age-dependent development of activity toward certain substrates [17, 18]. The demonstration that enzyme inducers such as sodium phenobarbital (PB) and 3-methylcholanthrene have selective stimulatory effects on microsomal glucuronyl transferase activities [3, 10] also supports the concept of multiple glucuronyl transferase.

In view of the many factors influencing liver microsomal glucuronyl transferase activity, we compared the glucuronidation of bilirubin and several steroids by rat liver microsomes. The results of this study indicate that the conjugation of bilirubin, testosterone, estradiol, estrone and estriol is differentially affected by such factors as addition of detergents to microsomal enzyme preparations and PB treatment of the animals. Furthermore, differences in the ability of liver microsomes from homozygous Gunn rats to conjugate bilirubin and several steroids are demonstrated.

METHODS

Animals. Long Evans male and female rats (Blue Spruce Farms, Altamont, N.Y.), male heterozygous Gunn rats, and male and female homozygous Gunn rats were fed a commerical diet (Purina Lab Chow) and water *ad lib.* PB was administered intraperitoneally to 130–140 g Long Evans rats at a dose of 75/mg/kg/day for 3–4 days. In long-term experiments, 45–50 g Long Evans rats received PB (0·1%) in their drinking water for 20 days. This concentration of PB resulted in an average daily dose of 175 mg/kg. Male and female homozygous Gunn rats (200–400 g) received PB (0·05%) in their drinking water for the same time period. The average daily dose of PB in these animals was 100 mg/kg.

Preparation of microsomes. Liver microsomes were prepared from 33% homogenates in 0·05 M Tris buffer (pH 7·7) containing 1·15% KCl as previously described [19]. The microsomal pellets were resuspended in

1·15% KCl and recentrifuged to ensure complete removal of hemoglobin and the soluble fraction. The final microsomal pellets were layered with 0·02 M Tris buffer (pH 7·5) and stored frozen for 1–7 days. Storage of microsomal pellets in this manner does not result in any significant loss of glucuronyl transferase activity. Before use, the microsomal pellets were thawed and suspended in 0·02 M Tris buffer (pH 7·5) to a concentration equivalent to 1 g liver (wet wt) per ml. Except where noted, the microsomal suspensions were mixed with equal volumes of 0·02 M Tris buffer (pH 7·5) or activated by mixing with equal volumes of the appropriate digitonin suspensions in Tris buffer. A 1% digitonin suspension was used for estrone and estriol studies, a 4% suspension was used for testosterone and estradiol, and a 7·5 per cent suspension was used for bilirubin. Further dilutions of all microsomal suspensions were made with 0·02 M Tris buffer (pH 7·5).

Steroid glucuronidation. Unactivated or digitonin-activated microsomal suspensions were incubated at 37 with the various ^{14}C-labeled steroids (0·13 mM) dissolved in methanol, UDPGA (5 mM) and $MgCl_2$ (5 mM) in a final volume of 1·0 ml of 0·05 M Tris buffer (pH 8·0). The studies were done under conditions where the formation of steroid glucuronides was proportional to tissue concentration and incubation time. In estrone and estriol studies, microsomes from 50 mg liver (wet wt) were used. The incubation times were 30 and 15 min for unactivated and activated microsomes respectively. In testosterone and estradiol studies, unactivated microsomes (25 mg liver, wet wt) and activated microsomes (2·5 mg liver, wet wt) were used, and the incubation time was 15 min. The protein concentration in liver microsomes of control rats was 16–18 mg/g of liver (wet wt). The increase in microsomal protein resulting from PB treatment of the animals is described later under "Protein determination."

The rate of glucuronidation of the various steroids was measured by the method of Miller *et al.* [20] with some modification. After incubation, unconjugated testosterone, estradiol or estrone was extracted twice for 15 min with 5 ml toluene in 15-ml shaking tubes. Unconjugated estriol was removed by three 15-min extractions with ether. After each extraction, the tubes were immersed in a dry ice–acetone bath to freeze the aqueous phase. The organic phase was removed each time and, after the last extraction, the aqueous phase was acidified with 0·25 ml of 4 N HCl. Five ml of a methylene chloride–isopropanol H_2O (75:25:2) solvent mixture was added to each tube, and the glucuronides were extracted by shaking for 15 min. A 2-ml portion of the organic phase was transferred to a scintillation vial and the radioactivity was quantified.

Bilirubin glucuronidation. A solution of bilirubin was prepared as previously described by Potrepka and Spratt [21] by rapidly dissolving 9·5 mg in 2·5 ml of 0·2 N NaOH. This was followed by the addition of 7·13 ml water. Unactivated and digitonin-activated microsomes equivalent to 50 mg liver (wet wt) were incubated at 37° for 30 and 15 min, respectively, with bilirubin (0·28 mM), UDPGA (5 mM) and $MgCl_2$ (5 mM) in a final volume of 0·5 ml of 0·05 M Tris buffer (pH 7·4). The method used for measuring the bilirubin glucuronide formed was essentially that described by

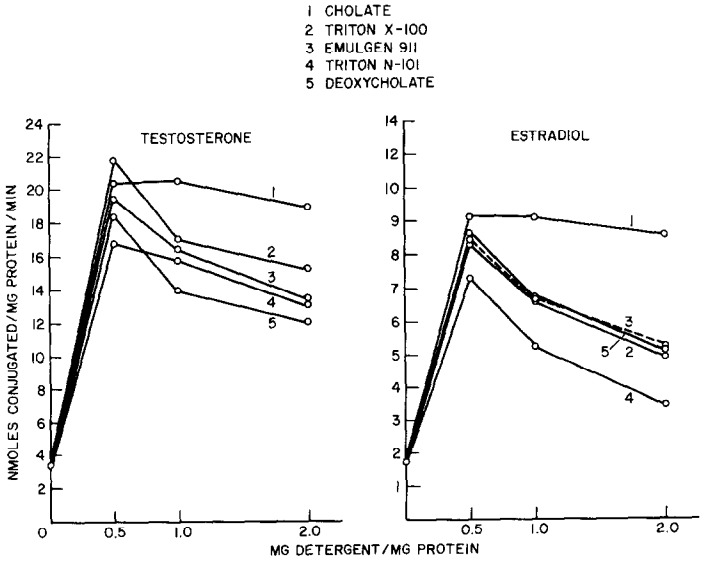

Fig. 1. Effect of various detergents on steroid glucuronidation by rat liver microsomes. Male rats (130–140 g) received PB (75 mg/kg, i.p.) for 3 days. The detergents were added to liver microsomes in specific ratios of mg detergent/mg microsomal protein. Unactivated microsomal suspensions (0·5 mg protein) or detergent-treated microsomal suspensions (0·05 mg protein) were incubated with ^{14}C-testosterone or estradiol (0·13 mM), UDPGA (5 mM) and $MgCl_2$ (5 mM) at 37 for 15 min. The glucuronides were quantified as described under Methods. 1, Cholate; 2, Triton X-100; 3, Emulgen 911; 4, Triton N-101; 5, deoxycholate.

Black *et al.* [22] with slight modification. The incubation was terminated by adding 1 ml glycine-HCl buffer (pH 2·8) to the incubation tubes, mixing the contents, and then transferring the tubes onto ice. After removal from the ice and allowing the samples to reach room temperature, 1 ml of freshly prepared diazo reagent was added. (This reagent was prepared by finely dispersing 0·1 ml ethyl anthranilate in 10 ml of 0·15 M HCl and then adding, with shaking, 0·3 ml of 0·5% $NaNO_2$ to the mixture.) The tubes were incubated for 30 min at 37° and the diazotization reaction was terminated by adding 0·5 ml of freshly prepared 7% ascorbic acid. The samples were then extracted with 5 ml of a 2-pentanone (redistilled)–*n*-butyl acetate (85:15) mixture by shaking for 30 min. The azo-pigment-containing upper layer was pipetted into cuvettes and the absorbance at 530 nm was determined spectrophotometrically against a solvent mixture blank. The amount of bilirubin conjugated was calculated from the difference between the two readings by use of the molar extinction coefficient ($44{\cdot}4 \times 10^3\ M^{-1}\ cm^{-1}$) as was previously determined [23].

Protein determination. Liver microsomal protein was determined by the method of Sutherland *et al.* [24]. In the studies reported below, treatment of Long Evans rats with PB (75 mg/kg/day, i.p.) once daily for 3 days resulted in a 15 per cent increase in microsomal protein per g wet wt of liver, while treatment for 20 days with 0·1% PB in the drinking water resulted in a 45 per cent increase. In the Gunn rat, a 20 per cent increase in microsomal protein per g wet weight occurred as a result of long-term PB treatment (0·05% PB in the drinking water for 20 days).

RESULTS

Effect of various detergents on steroid glucuronyl transferase activity in rat liver microsomes. Several detergents were tested for their ability to increase the glucuronidation of testosterone and estradiol by rat liver microsomes. Various amounts of the detergents expressed as mg detergent per mg of microsomal protein were combined with the microsomal suspensions prior to incubation with substrate. The activation profiles for cholate, Triton X-100, Emulgen 911, Triton N-101 and deoxycholate are shown in Fig. 1 and indicate that maximal stimulation of testosterone and estradiol glucuronidation occurred in each case at ratios of 0·5 mg detergent per mg of protein. Varying degrees of inhibition were observed at higher concentrations for all the detergents except cholate, which was the least inhibitory.

Effect of digitonin on the glucuronidation of bilirubin and various steroids by rat liver microsomes. Hepatic bilirubin glucuronidation has been reported to be increased by treatment of enzyme preparations with digitonin [22, 25, 26]. In our studies the maximal increase in bilirubin conjugation occurred when 5–10% digitonin suspension was mixed with an equal volume of a microsomal suspension.

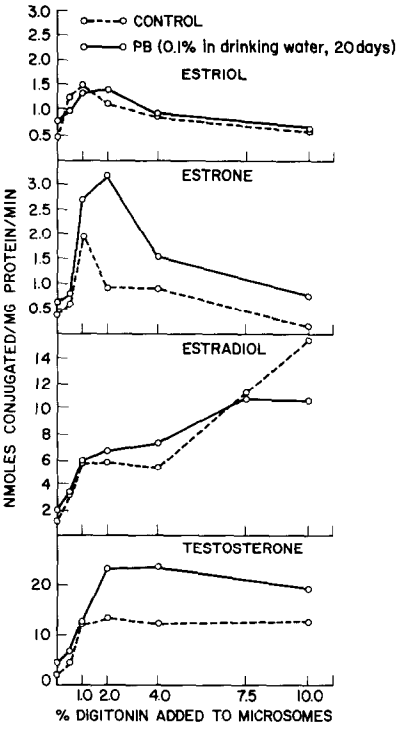

Fig. 2. Effect of digitonin on steroid glucuronidation by rat liver microsomes. Male rats received 0·1% PB in their drinking water for 20 days. Liver microsomal suspensions (equivalent to 1 g wet wt liver/ml) were combined with equal volumes of 0·5 to 10% digitonin suspensions in 0·02 M Tris buffer (pH 7·5). Incubation conditions and the assay for the glucuronides formed are described under Methods.

Glucuronidation of testosterone, estradiol, estrone and estriol by liver microsomes from control and PB-treated rats was also measured as a function of digitonin concentration. The results shown in Fig. 2 indicate that there are marked differences in the extent of activation and the concentration of digitonin needed for maximal activation of these steroids. Estriol, estrone and testosterone were maximally conjugated by microsomes from control rats when a 1% digitonin suspension was added to an equal volume of a microsomal suspension. The conjugation of estriol and estrone was inhibited at higher concentrations. When suspensions with a digitonin concentration greater than 4 per cent were added to microsomes, estradiol conjugation was markedly enhanced. Microsomes from PB-treated rats generally showed maximal conjugating activity at slightly higher concentrations of digitonin than did control microsomes, except in the case of estradiol conjugation where activity at the highest concentration of digitonin was lower in microsomes by PB-treated animals than in microsomes from the controls.

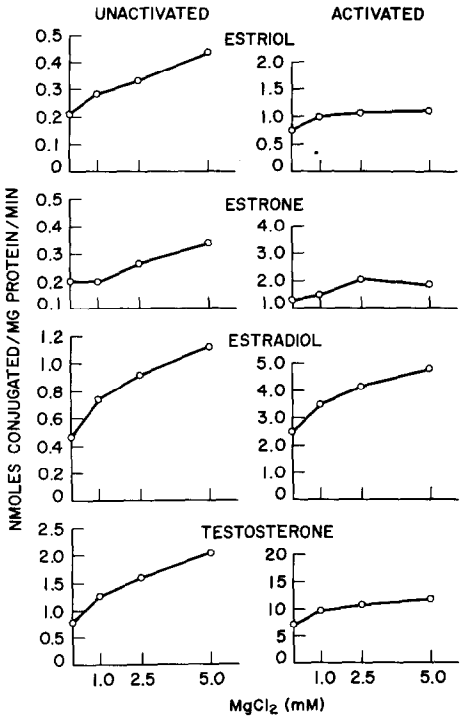

Fig. 3. Effect of Mg^{2+} on liver microsomal steroid glucuronidation. Liver microsomes from male rats weighing 130 g were activated with the appropriate digitonin suspensions as described under Methods. A 1% digitonin suspension was used for estrone and estriol studies, and a 4% digitonin suspension was used for testosterone and estradiol studies. Unactivated and activated microsomal suspensions were incubated with the various substrates and UDPGA in the presence of the indicated concentrations of $MgCl_2$.

Effect of Mg^{2+} concentration on steroid glucuronidation by rat liver microsomes. Divalent cations, such as Mg^{2+} and Ca^{2+}, have been shown to affect differentially the glucuronidation of various substrates [5, 12–14]. Some investigators have reported a requirement for these metals, while others have demonstrated inhibitory effects. In fact, the Mg^{2+}-induced increase in the rate of synthesis of bilirubin glucuronide was shown to be the result of an enhancement of bilirubin solubility and not the result of a direct effect on kinetic behavior of the glucuronyl transferase, as is the case with other substrates [13].

In view of these differential effects of the divalent cations on glucuronidation, we thought it important to study their effects on the glucuronidation of the various steroids. The rate of steroid glucuronidation by unactivated microsomes was measured as a function of Mg^{2+} concentration in the incubation mixture. The results shown in Fig. 3 indicate a partial requirement for Mg^{2+} in steroid glucuronidation.

Effect of treatment of rats with PB for 3 days on bilirubin and steroid glucuronidation. Rats were pretreated with PB (75 mg/kg/day, i.p.) once daily for 3 days. Twenty-four hr after the last injection, the animals were killed and liver microsomes were prepared and incubated with the various substrates and UDPGA as described under Methods. The results obtained with both unactivated and digitonin-activated microsomes are shown in Fig. 4. Significant increases in testosterone and estradiol glucuronidation were observed with both activated and unactivated microsomes from the PB-treated rats. PB pretreatment had no stimulatory effect, however, on estrone or bilirubin conjugation when the data were expressed per mg of microsomal protein.

Effect of treatment of rats with PB for 20 days on bilirubin and steroid glucuronidation. Male rats received 0·1% PB in their drinking water for 20 days. Bilirubin and steroid glucuronyl transferase activities in activated and unactivated liver microsomes were determined and the results are shown in Fig. 5. A significant stimulatory effect of PB on bilirubin glucuronidation was observed only when activated microsomes were used, while a decrease occurred with unactivated microsomes. Other investigators have also reported a stimulatory effect of long-term PB treatment on liver microsomal bilirubin glucuronidation as compared to

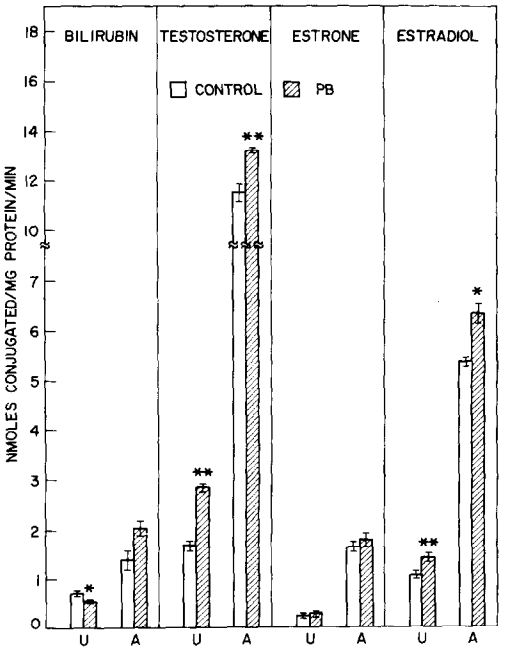

Fig. 4. Effect of short-term PB treatment on glucuronyl transferase activity of rat liver microsomes. Male rats (130–140 g) received PB (75 mg/kg, i.p.) once daily for 3 days. The digitonin activation of liver microsomes and the incubation conditions are described under Methods. Unactivated and activated microsomes are indicated by U and A respectively. Each value is the average ± S. E. obtained from three determinations (three rats per determination). * = $P < 0.05$; ** = $P < 0.01$.

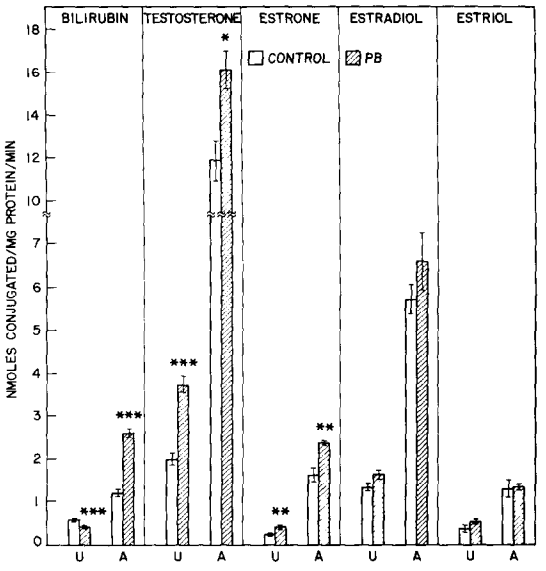

Fig. 5. Effect of long-term PB treatment on glucuronyl transferase activity of rat liver microsomes from male rats. Rats (45–50 g) received 0·1% PB in their drinking water for 20 days. The digitonin activation of liver microsomes and the incubation conditions are described under Methods. Unactivated and activated microsomes are indicated by U and A respectively. Each value is the average ± S. E. obtained from three determinations (three rats per determination). * = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.01$.

the lack of effect when PB is administered for a short period of time [2, 27].

A significant stimulatory effect of PB on testosterone and estrone glucuronidation was observed with both activated and unactivated microsomes (Fig. 5). In contrast to the results in Fig. 4 showing an increase in estradiol glucuronidation after treatment with PB for 3 days, no significant increase was observed after long-term PB treatment. Estriol glucuronidation was not increased by the 20-day treatment with PB. Similar effects of PB on the glucuronidation of bilirubin and the steroids were obtained when liver microsomes from female rats were used (Fig. 6).

When the data from Fig. 5 (nmoles substrate conjugated/mg protein/min) were expressed as μmoles substrate conjugated/g liver/hr, a significant stimulatory effect of PB on the glucuronidation of bilirubin, testosterone, estrone, estradiol and estriol was observed (Fig. 7). Increases in the glucuronidation of all the substrates by female rat liver microsomes were also observed when the data from Fig. 6 were similarly recalculated. These increases in glucuronidation are related to the increase in total microsomal protein per g of liver caused by treatment of rats with PB.

Steroid glucuronidation by liver microsomes from homozygous Gunn rats. Although homozygous Gunn rats are genetically deficient in the ability to conjugate bilirubin, they are able to conjugate other substrates such as *p*-nitrophenol and chloramphenicol [1,3]. It was, therefore, of interest to determine whether liver microsomes from Gunn rats could metabolize steroids by glucuronidation. The results of this study (Table 1) show that marked differences occurred in glucuronidation by Gunn rat liver microsomes as compared to glucuronidation by liver microsomes from normal or heterozygous Gunn rats. Although the ability to conjugate bilirubin is absent in liver microsomes from the homozygous Gunn rat, the rate of testosterone glucuronidation was only 21 per cent lower than in control rats, and the rate of estrone glucuronidation was 48 per cent lower (Table 1A).

An apparent absence of a digitonin-induced increase in estrone glucuronidation by homozygous Gunn rat liver microsomes was observed. This absence of digitonin activation resulted in a magnification of the difference in enzyme activity (after digitonin treatment) between microsomes from Gunn rats and microsomes from control rats (Table 1B). The digitonin activation of testosterone glucuronidation was the same for both the homozygous Gunn rat and the control rat. Thus, in this case, the per cent difference in enzyme activity between the control and homozygous Gunn rat was not appreciably changed by digitonin.

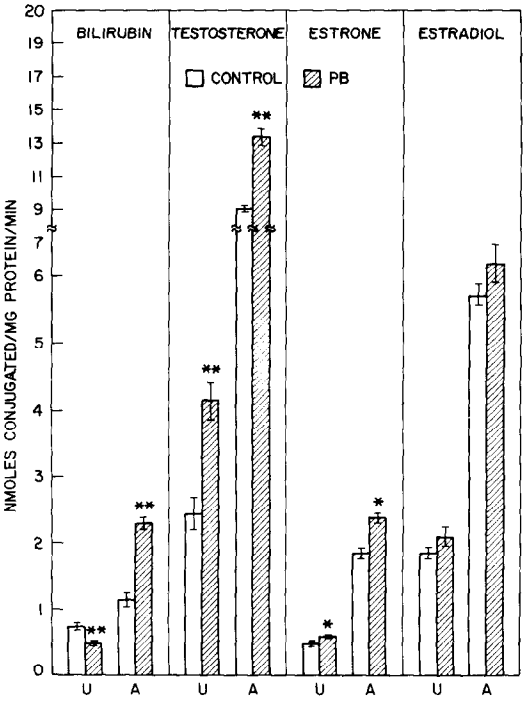

Fig. 6. Effect of long-term PB treatment on glucuronyl transferase activity of rat liver microsomes from female rats. Treatment of the rats and digitonin activation of liver microsomes are the same as in Fig. 5. Unactivated and activated microsomes are indicated by U and A respectively. * = $P < 0.02$; ** = $P < 0.01$.

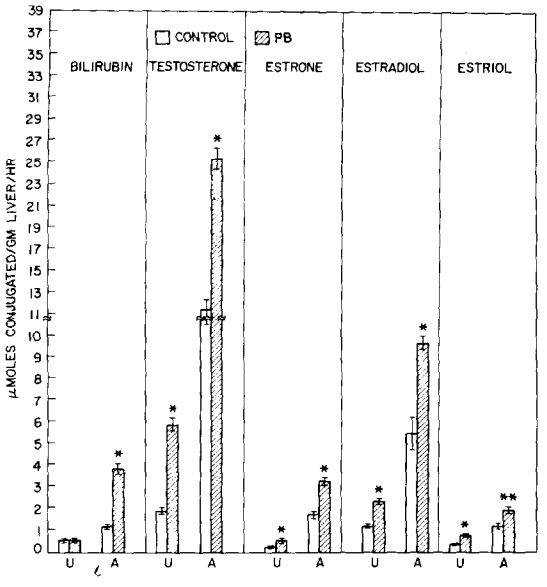

Fig. 7. Effect of long-term PB treatment on liver microsomal glucuronyl transferase activity per g wet weight liver obtained from male rats. The conditions are the same as those in Fig. 5. Unactivated and activated microsomes are indicated by U and A respectively. Activity is calculated as μmoles substrate conjugated/g liver (wet wt)/hr. * = $P < 0.01$; ** = $P < 0.02$.

To confirm the formation of testosterone glucuronide by liver microsomes from homozygous Gunn rats, the method of Miller *et al.* [20] for estrone and estradiol was used with some modification. The radioactive metabolites which were not extracted with toluene but which were extracted into the methylene chloride–isopropanol–H_2O (75 : 25 : 2) mixture after incubation of ^{14}C-testosterone with liver microsomes of homozygous Gunn rats were reincubated at 37° in 2 M acetate buffer with β-glucuronidase at pH 5·0 for 18 hr. This incubation resulted in a marked increase in radioactivity which was extractable into toluene and represented unconjugated testosterone (Table 2). Incubation of the metabolites with β-glucuronidase in the presence of saccharic acid 1,4-lactone, a specific inhibitor of the enzyme, resulted in the recovery of much less toluene-extractable radioactivity, but a substantial amount of radioactivity extractable into the polar methylene chloride–isopropanol–H_2O mixture. Thus, the conversion of the polar conjugated products of testosterone glucuronidation to unconjugated testosterone by β-glucuronidase was inhibited by saccharic acid 1,4-lactone. These findings indicate that testosterone glucuronide is indeed formed during the incubation of homozygous Gunn rat liver microsomes with testosterone and UDPGA.

Table 1. Glucuronyl transferase activity of liver microsomes from normal or heterozygous Gunn and homozygous Gunn male rats

	Normal or heterozygous Gunn	Homozygous Gunn	% of control
	(nmoles/mg protein/min)		
Unactivated microsomes			
Testosterone	1·99	1·57	79
Estrone	0·25	0·13	52
Bilirubin		0	
*Activated microsomes**			
Testosterone	6·37	4·60	72
Estrone	0·65	0·19	29
Bilirubin	0·35	0	0

* Liver microsomal suspensions (1 g wet wt liver/ml) were mixed with equal volumes of the appropriate digitonin suspension (1% for estrone, 4% for testosterone and 7·5% for bilirubin). Each value is the average obtained using two to three rats.

Effect of PB on glucuronyl transferase activity of liver microsomes from homozygous Gunn rats. Male and female homozygous Gunn rats received 0·05% PB in their drinking water for 20 days. Although PB administration did not enhance the conjugation of bilirubin by liver microsomes in the Gunn rat, varying increases in liver microsomal steroid glucuronidation were observed after PB treatment, with testosterone and estrone glucuronidation showing the most significant increases (Table 3).

DISCUSSION

Several studies have suggested that there are a multitude of liver UDP glucuronyl transferases with different substrate specificities [1–18]. Although bilirubin conjugation has been studied extensively, relatively few comparative studies have dealt with the glucuronidation of steroid hormones and other normal body constituents.

The studies described in this paper indicate that the glucuronidation of bilirubin and several steroids by rat liver microsomes is differentially affected by a number of factors such as detergent treatment of microsomal preparations, administration of PB to the rats, and the source of the liver microsomes (homozygous Gunn versus control rats). The extent of the detergent-induced increase in the glucuronidation of bilirubin and the steroid hormones was substrate dependent. The curves for glucuronidation activity versus concentration of digitonin differed for bilirubin, estradiol, estrone, estriol and testosterone. The ability of detergent treatment of enzyme preparations to increase the UDPGA-dependent conjugation of many substrates *in vitro* has been well documented [20, 22, 25, 26, 28]. Our data showing that Trition X-100 enhances the microsomal glucuronidation of estradiol, however, are in contrast to those of Miller *et al.* [20], who could not demonstrate this effect with estradiol as the substrate. Perhaps the ratio of detergent concentration to microsomal protein concentration used in their studies was too low for maximal activation. In contrast to the enhancement of bilirubin and steroid glucuronidation by treatment of liver microsomes with digitonin, the 6β-, 7α- and 16α-hydroxylation of testosterone by the mixed-function oxidase system was inhibited by 60 per cent after this treatment (unpublished observations).

Table 2. Evidence for testosterone glucuronide formation by liver microsomes from the homozygous Gunn rat*

Treatment	Radioactivity as nmoles of testosterone extractable into	
	Toluene	Methylene chloride–isopropanol–H_2O
None	0	7·03
β-Glucuronidase (3000 units)	6·83	0·20
β-Glucuronidase + saccharic acid 1,4-lactone (10^{-3}M)	2·16	4·87

* Testosterone-4-^{14}C (123 nmoles) was incubated for 30 min with digitonin-activated microsomes from 5 mg liver from homozygous Gunn rats. After removal of the substrate by two extractions with toluene, the metabolites were extracted into methylene chloride–isopropanol–H_2O (75:25:2). Two ml of the extract containing the metabolites was evaporated to dryness and the residue was dissolved in 2 M acetate buffer, pH 5·0. The appropriate additions were made and the mixtures (1·0 ml final volume) were incubated for 18 hr at 37°. Following two toluene extractions, 0·25 ml of 4 N HCl was added to each tube and the contents were extracted with methylene chloride–isopropanol–H_2O (72:25:2). The total toluene-extractable radioactivity and that extractable into the methylene chloride–isopropanol–H_2O mixture was measured.

Table 3. Effect of long-term phenobarbital treatment on glucuronyl transferase activity of unactivated liver microsomes from homozygous Gunn rats

Rats*	Bilirubin	Testosterone	Estrone	Estradiol	Estriol
		(nmoles/mg protein/min)			
Male					
Control	0	1·39	0·23	0·72	0·30
PB	0	2·80	0·42	1·28	0·44
Female					
Control	0	1·40	0·24	0·96	0·31
PB	0	2·72	0·46	1·40	0·49

* Rats received 0·05% phenobarbital in their drinking water for 20 days. Each value was obtained using two to three rats.

Other investigators have also demonstrated an inhibitory action of detergents on liver microsomal mixed-function oxidases [29–32].

The data presented here and elsewhere [20] indicate that the rate of estradiol glucuronidation by rat liver microsomes was higher than that of estrone. Although these data suggest the possibility that the alcohol hydroxyl group in the 17β-position may be conjugated in the rat to a greater extent than the phenolic hydroxyl group in the 3-position. Breuer and Wessendorf [33] demonstrated that in the rabbit the glucuronidation of estradiol occurs primarily at the 3-position. Furthermore, estradiol-3-methyl ether is conjugated by rabbit liver microsomes to a lesser extent than is estrone [34]. It appears, therefore, that species differences exist in the rate of conjugation of the phenolic and alcoholic hydroxy groups in the 3- and 17β-positions, respectively, and/or that modification of the molecule at the 17β-position may influence glucuronidation at the 3-position. It was of interest that pregnenolone and corticosterone, steroids which contain alcoholic hydroxyl groups in positions other than the 17-position, were not conjugated to any appreciable extent by rat liver microsomes (unpublished observations). Interestingly, in our studies, estriol, which has an alcoholic hydroxyl group in the 16α-position in addition to hydroxyl groups in the 3- and 17β-positions, was conjugated by rat liver microsomes more slowly than was estradiol and to the same extent as estrone. Slaunwhite *et al.* [35] have shown that the conjugation of radioactive estriol by human liver microsomes is inhibited by addition to the incubation of unlabeled estrogens hydroxylated at the 16α-position, but not by the 3α-hydroxysteroids, 16-epiestriol or estradiol. These data suggest that estriol may undergo glucuronidation by a glucuronyl transferase different from the transferase(s) that conjugates certain other steroids.

The administration of PB to rats for 3 days caused a small but significant increase in microsomal testosterone and estradiol glucuronidation, but had no stimulatory effect on estrone or bilirubin glucuronidation when the data obtained with digitonin-activated microsomes were expressed as amount of glucuronidation per mg of microsomal protein. When rats were treated with PB over the course of 20 days, a different pattern of enzyme induction emerged. The stimulatory effect of PB on testosterone glucuronidation in this study was similar to that obtained after short-term treatment. However, estrone conjugation per mg of microsomal protein was induced, while estradiol and estriol conjugation was unaffected. An induction of bilirubin glucuronidation was observed after the 20-day PB treatment only when digitonin-activated microsomes were used. When unactivated microsomes were incubated, the same decrease in specific enzyme activity occurred as in short-term PB experiments. The absence of an in-

ductive effect of PB on bilirubin conjugation by unactivated microsomes might be attributed to a limited ability of the bilirubin to reach a relatively nonaccessible glucuronyl transferase in the intact microsomal membrane. Other investigators have reported that PB does not stimulate the glucuronidation of *p*-nitrophenol unless activated enzyme preparations are used [36, 37].

Although liver microsomes from homozygous Gunn rats are totally deficient in the ability to conjugate bilirubin, these microsomes are able to conjugate testosterone, estrone, estradiol and estriol. The rate of estrone conjugation *in vitro* was substantially lower in these animals than in heterozygous nonjaundiced Gunn or normal rats, while the rate of testosterone conjugation was only slightly lower. Furthermore, digitonin treatment of microsomes from homozygous Gunn rats did not increase the rate of estrone conjugation but did increase the rate of testosterone conjugation to the same extent as in heterozygous Gunn or normal rats. Zakim *et al.* [38] have demonstrated that phospholipase A and Triton X-100 have no effect on microsomal *p*-nitrophenol or *o*-aminophenol glucuronidation in the homozygous Gunn rat and have suggested that there may be a defect in microsomal phospholipid structure in this animal. Although this possible defect might explain the lack of a digitonin effect on estrone conjugation, it is apparently not a determining factor in the ability of the Gunn rat to conjugate testosterone. Drucker [39] has shown that another steroid aglycone, tetrahydrocortisone, is conjugated normally by homozygous Gunn rat liver microsomes.

The administration of PB to homozygous Gunn rats for 20 days was also shown in this paper to increase testosterone and estrone glucuronidation significantly, but in agreement with studies by other investigators [2, 40], the metabolism of bilirubin was not stimulated.

The many differences in liver microsomal glucuronidation of the various substrates described in this paper support the concept that a multitude of glucuronyl transferases exist in liver microsomes. The results of these studies suggest that the glucuronyl transferase for bilirubin is different from the enzyme(s) that metabolizes testosterone, estradiol, estrone and estriol by glucuronidation. However, direct evidence for multiple glucuronyl transferases will only emerge from the solubilization and purification of each of these enzymes.

Acknowledgement—We thank Dr. R. Swarm (Department of Toxicology and Experimental Pathology, Hoffmann-La Roche Inc.) and Dr. I. Arias (Albert Einstein College of Medicine) for Gunn rats, and we also thank Mrs. Cathy Chvasta for her assistance in the preparation of this manuscript.

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