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Effect of Albumin on the *in Vitro* Conjugation of Bilirubin by Rat Liver Microsomes (41883)

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Abstract. These studies were carried out to determine whether bovine serum albumin (BSA), which is usually included in the incubation mixture for the *in vitro* determination of bilirubin-UDP-glucuronyl transferase (GT) activity, affects GT activity. Using bilirubin as substrate, addition of BSA to the enzyme reaction mixture at concentrations varying from 2 to 30 mg/ml resulted in a dose-related inhibition of "native" GT activity of rat liver microsomes. When detergent-activated enzyme was employed, increasing concentrations of BSA also required higher concentrations of deoxycholate, digitonin, or Triton X-100 to produce maximal bilirubin conjugation. Low BSA concentrations (2 mg/ml) prevented enzyme activation by both detergents and UDP-*N*-acetyl glucosamine. When BSA was omitted and bilirubin dissolved in dimethyl sulfoxide, UDP-*N*-acetyl glucosamine failed to enhance GT activity, and activation by detergents was only 15–25% of that observed in the presence of optimal concentrations of BSA. When rat albumin was substituted for BSA, a similar dose-related inhibition of *in vitro* bilirubin conjugation by untreated microsomes was observed, although at any given albumin concentration, GT activity was lower with rat than with bovine albumin. Additionally, both detergents and UDP-*N*-acetyl glucosamine produced similar GT activation regardless of the rat albumin concentration. Finally, these effects of BSA and rat albumin could not be reproduced when β-lactoglobulin was employed and/or when *p*-nitrophenol was the acceptor substrate of GT. These findings indicate that albumin, in particular BSA, profoundly and selectively influences the *in vitro* activity of microsomal GT toward bilirubin as the acceptor substrate.

UDP-glucuronyl transferase (GT) denotes the group of enzymes responsible for the addition of glucuronic acid to a variety of aglycones of endogenous and exogenous origin. Accordingly, the *in vitro* activity of GT can be measured using a number of acceptor substrates, and several systems have been described for its assay (1–8).

A specific bilirubin GT has been isolated, and detailed studies of its enzymologic properties have been reported (9). Nevertheless, in certain clinical settings, especially in puzzling cases of unconjugated hyperbilirubinemia, assay of bilirubin GT is still frequently performed employing crude homogenates of biopsy samples of liver tissue (10) or, when larger samples are available, partially purified microsomal preparations (1, 2). Several authors have noted the wide variation in assay results produced by superficially similar methods (1, 2, 10), and the apparent discrepancy in many situations between the bilirubin GT activity and the serum concentration of unconjugated bilirubin (11, 12). The reasons for the discrepancies have not been fully elucidated.

When bilirubin is employed as the acceptor substrate, determination of GT activity usually involves the introduction into the enzyme incubation mixture of a certain amount of albumin (4–8 mg/ml), most often bovine serum albumin (BSA) (1, 2, 10). This is required because at the optimal pH of the enzyme reaction (7.7 at 37°C (1, 2, 10, 13)), bilirubin is virtually insoluble in the aqueous phase (14, 15) and must be bound to albumin to avoid precipitation. However, despite this commonly used procedure, no studies have systematically examined whether the presence of albumin influences the enzyme activity. In this report we describe the effects of albumin on the *in vitro* conjugation of bilirubin by rat liver microsomes. Similar considerations may apply to the assay of human bilirubin GT, although limited access to fresh human liver tissue has thus far prevented direct confirmation of these findings in man.

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Methods. *Chemicals and solutions.* All chemicals including proteins used in these studies were reagent grade and, unless otherwise indicated, were purchased from Sigma (St. Louis, Mo.). Bovine serum albumin (BSA) was fatty acid free as defined by the supplier. The original source of β-lactoglobulin (folate binding protein, containing both A and B fractions) was cow's milk. Rat albumin was Fraction V. Proteins were generally used as furnished by the supplier. However, in selected studies, they were first dialyzed for 18 hr at 4°C against 20 vol of 0.1 *M* sodium bicarbonate, pH 8.6. Most reagent solutions for the GT assay were prepared immediately before use; the reader is referred to our previous report for detailed information (13). Bilirubin (E_{450} = 59,000–61,000; >92% IXα by thin layer chromatography (16)) was dissolved first in a few drops of NaOH (0.25 *M*) and subsequently added to a solution of BSA, rat albumin, or β-lactoglobulin. The resulting stock solution contained the desired concentration of unconjugated bilirubin (0.856 m*M* or 0.5 mg/ml for standard enzyme assay) and 8 mg/ml of one of the above-mentioned proteins. This solution was adjusted to a pH of 7.9 at 22°C (7.7 at 37°C) and was invariably used within 6 hr of preparation, over which it was stable with no evidence of either precipitation or colloid formation. When unconjugated bilirubin was dissolved in dimethyl sulphoxide (DMSO) (Fisher, Pittsburgh, Pa.), the pigment was added directly to the solvent to achieve a concentration of 0.856 m*M*. Chromatographically pure bilirubin diglucuronide was isolated from rat bile as previously described (17), stored *in vacuo* and in the dark at −20°C, and dissolved just prior to use. All the procedures for the preparation of the bilirubin solutions as well as those for measuring GT activity were carried out in the presence of subdued light.

Animals and microsome preparation. Male Sprague–Dawley rats (260–280 g) were purchased from Perfection Breeders (Douglasville, Pa.) and kept for at least 1 week in a temperature-controlled room (22°C) with alternating 12-hr light–dark cycles prior to being used. Except where otherwise noted, hepatic microsome fractions were prepared in 0.25 *M* sucrose containing 1 m*M* EDTA (pH 7.4) by a conventional ultracentrifugation procedure as reported in detail elsewhere (13). Microsome pellets were stored at −20°C and resuspended in an appropriate volume of sucrose–EDTA so that the microsomes isolated from 1 g of liver were contained in a 4-ml suspension. For a limited number of comparison studies, microsomes prepared, stored, and resuspended in 150 m*M* KCl (2) were employed.

Enzyme activation. GT activity toward bilirubin or *p*-nitrophenol as the acceptor substrate was measured in microsome suspensions in which the enzyme was either in its "native" form or activated with a variety of detergents or with the sugar nucleotide UDP-*N*-acetyl glucosamine (UDPNAG). The source of "native" enzyme was a suspension of microsomes obtained from 1 g of liver in 8 ml of sucrose–EDTA, which was obtained by diluting 1 vol of the original suspension (4 ml) with 1 vol of sucrose–EDTA. Similarly, the detergent-activated enzyme was prepared by adding 1 vol of the original microsome suspension to 1 vol of sucrose–EDTA containing the desired concentration of the detergent used. Both untreated and detergent-treated microsome suspensions were incubated at 0°C for 30 min before being added to the reaction mixture. When GT was activated by UDPNAG, the latter was added directly to the reaction mixture.

GT activity determination. The activity of GT was measured aerobically at 37°C using either bilirubin or *p*-nitrophenol as the acceptor substrate. When bilirubin was employed, the standard incubation mixture contained: Tris (pH 7.7 at 37°C), 250 m*M;* MgCl, 10 m*M;* UDP-glucuronic acid, 5.17 m*M;* microsomal protein, 0.25–0.35 mg; bilirubin, 0.214 m*M;* and the desired concentration of BSA, rat albumin, or β-lactoglobulin. When applicable, UDPNAG was included at the desired concentration (see below). In studies omitting BSA, rat albumin, and β-lactoglobulin, bilirubin was dissolved in DMSO, at a final concentration in the incubation mixture of 2.5 *M*. Similar incubation mixtures were prepared when *p*-nitrophenol was the acceptor substrate, except that Tris, pH 7.4, was used instead of Tris, pH 7.9, and *p*-nitrophenol (0.75 m*M*) was substituted for bilirubin. In both assays, the final volume of the reaction mixture was 1 ml and all components were

added to a 10 ml Erlenmeyer flask which was kept on ice until incubation was initiated. In the bilirubin GT assay, incubations were done for 30 min, whereas in the *p*-nitrophenol GT assay, samples were incubated for 10 min. The activity of GT toward bilirubin and *p*-nitrophenol was measured by minor modifications of the methods of Strebel and Odell (2) and Isselbacher *et al.* (3), respectively, as previously described (18). Protein concentration was determined by the Lowry method (19) using BSA as standard.

Effect of BSA on the extraction and diazotization of conjugated bilirubin. Determination of GT activity by the procedure used in the present studies involves coupling of the conjugated bilirubin with diazotized sulfanilic acid, after its extraction from the unconjugated pigment by solvent partitioning (lactic acid–ethyl acetate:chloroform), according to a modification of the method of Weber and Schalm (20). The validity of applying this partition method to the extraction of bile pigments from tissue-derived preparations, such as the enzyme incubation mixture, previously demonstrated in our laboratory (21), was again confirmed; ≥91% of added radiolabeled bilirubin mono- and diglucuronides was invariably recovered in the polar, upper layer of the partition system, and >95% of labeled unconjugated bilirubin in the nonpolar, lower chloroform layer. Nevertheless, it was essential to ascertain that any observed effects of BSA on GT activity were not related to technical artifacts introduced into the extraction and/or diazotization procedures, but reflected real changes in enzyme activity. Accordingly, conversion of the measured extinction values into units of enzyme activity was made using as standards modified incubation mixtures which contained two different concentrations of BSA (2 or 18 mg/ml) and known amounts of pure bilirubin diglucuronide (17). UDP-glucuronic acid and UDPNAG were omitted and these preparations were kept on ice prior to determination of extinction.

Estimation of the influence of albumin on free bilirubin concentration. To examine the hypothesis that albumin influences GT activity simply through its effect on the concentration of free bilirubin in the reaction mixture, the free bilirubin concentrations present in solutions containing 0.214 m*M* total bilirubin and 0.029 m*M* to 0.441 m*M* bovine or rat albumin (bilirubin:albumin molar ratio 7.3–0.49) were estimated. The estimation was done using published bilirubin–albumin association constants for multiple binding sites, determined for rat and bovine albumin by various techniques (16, 22). Particular comparisons were made for binding constants obtained by a standard fluorescence technique (22), the only method for which BSA and rat albumin were studied under comparable conditions. Computation of the free bilirubin concentration was done employing the algorithm published by Wosilait and associates (23), adapted in MINC–BASIC for use on a MINC-11 digital minicomputer (Digital Equipment, Maynard, Mass.).

Results. *Validation of methods.* Irrespective of the albumin concentration in the reaction mixture, conjugated bilirubin formation increased linearly with time for at least 30 min whether native, detergent activated (digitonin, deoxycholate), or UDPNAG-activated microsomes were employed (Fig. 1). In addition, as we have reported previously, quantitation of conjugated bilirubin formation yielded identical results whether measured by the solvent extraction method of Weber and Schalm, or by selective diazotization with ethyl anthranilate at pH 2.7 (13). Glucuronidation of *p*-nitrophenol has also been shown to be linear over the initial 10 min of the reaction (18).

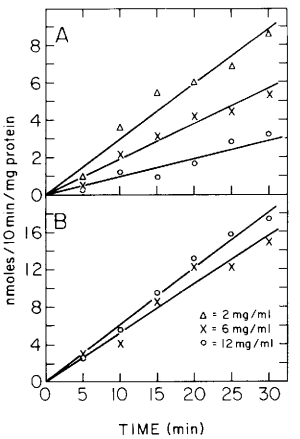

FIG. 1. Formation of conjugated bilirubin by native (A) and 0.1% deoxycholate-activated (B) rat liver microsomes at three different concentrations of albumin. Figure illustrates linearity of the reaction over the initial 30 min of incubation. Similar linearity was observed with digitonin- or UDPNAG-activated microsomes.

In view of the established linearly of the conjugation of both substrates investigated in this report, measurement of product formation at any point within the linear period provides equivalent information to determination of initial reaction rates.

Effect of bilirubin concentration on bilirubin GT activity. As already reported by other investigators (1, 2), increasing concentrations of unconjugated bilirubin in the enzyme incubation mixture produced a biphasic effect on the bilirubin glucuronidation rate: an initial increase followed by a decrease. As illustrated in Fig. 2, however, the activating effect of bilirubin was dependent on the amount of BSA present in the incubation mixture, and proportionally higher concentrations of bilirubin were necessary to produce maximal GT activity when increasing concentrations of BSA were included. Thus, in the presence of 2, 6, and 12 mg/ml BSA, the highest GT activity with digitonin-activated microsomes was obtained with 0.086, 0.214, and 0.342 m*M* bilirubin, respectively. This effect of BSA was similarly observed when "native" enzyme or enzyme activated by UDPNAG or deoxycholate was employed.

Effect of BSA on bilirubin GT activity. When a constant concentration of unconjugated bilirubin (0.214 m*M*) was included in the enzyme incubation mixture, the apparent activity of bilirubin GT in untreated microsomes ("native" enzyme) varied inversely as a logarithmic function of the BSA concentration. The highest GT activity, which ranged from 7.8 to 10.2 nmoles/10 min/mg protein, was observed with the lowest BSA concentration (2 mg/ml), and proportionally lower enzyme activities were obtained as the BSA concentration was increased up to 30 mg/ml (Fig. 3). With 30 mg/ml BSA, the rate of bilirubin glucuronidation averaged 1.25 nmoles/10 min/mg, a value which represented roughly 10–15% of that observed with 2 mg/ml BSA. Virtually identical data were obtained when dialyzed albumin was employed.

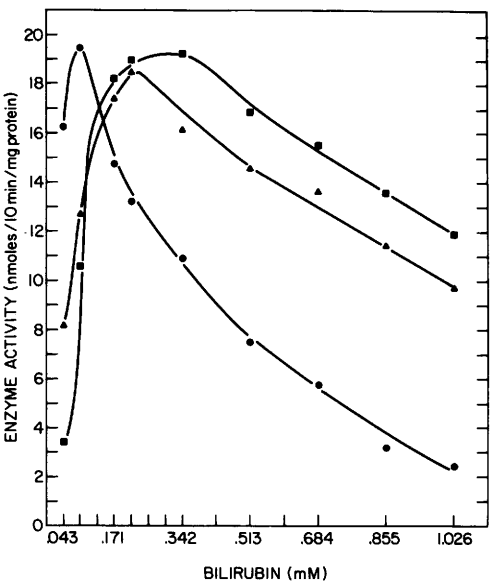

FIG. 2. Effect of unconjugated bilirubin on bilirubin GT activity in presence of 2 (●), 6 (▲), and 12 (■) mg/ml bovine serum albumin.

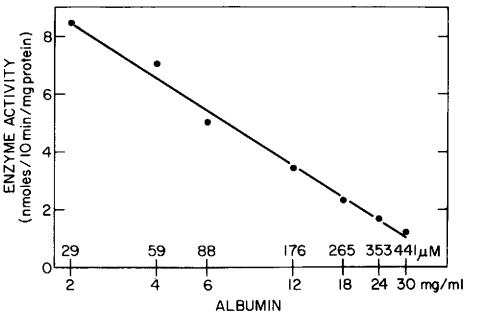

FIG. 3. Effect of bovine serum albumin on "native" bilirubin GT activity.

The concentration of BSA in the incubation mixture influenced not only the activity of the "native" GT, but also that of the detergent-activated enzyme. Figure 4 illustrates the GT activation profile by deoxycholate in the presence of 2, 6, and 18 mg/ml BSA. With 2 mg/ml BSA, little or no activation by deoxycholate was observed, and only an inhibitory effect on GT activity was obtained with concentrations of the detergent greater than 0.075–0.10%. Conversely, when BSA was included at a concentration of 6 mg/ml or greater, the typical biphasic effect of deoxycholate was observed, which consisted of an initial activation followed by inhibition of GT activity. However, as the presence of BSA also resulted in buffering the activating as well as the inhibitory effect of the detergent, proportionally higher concentrations of deoxycholate were necessary to produce maximal GT activation when increasing BSA concentrations were included. Thus, in the presence of 6, 18,

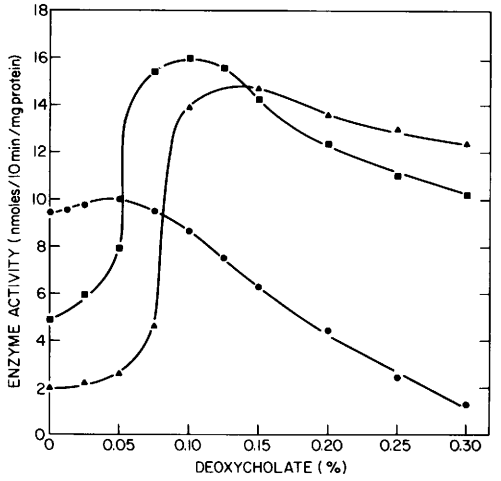

FIG. 4. Effect of deoxycholate on bilirubin GT activity in presence of 2 (●), 6 (■), and 18 (▲) mg/ml bovine serum albumin.

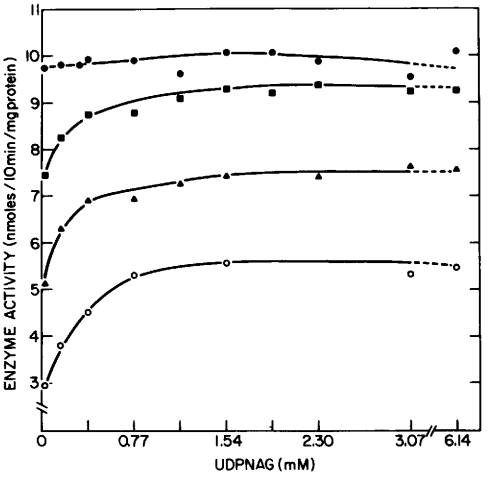

FIG. 5. Effect of UDPNAG on bilirubin GT activity in presence of 2 (●), 4 (■), 6 (▲), and 12 (○) mg/ml bovine serum albumin.

and 30 mg/ml BSA, maximal GT activity was obtained with 0.1, 0.15, and 0.2% deoxycholate, respectively. These effects of BSA on the activation profile of bilirubin GT by deoxycholate were similarly observed when other detergents, both neutral (digitonin) and cationic (Triton X-100), were employed.

When UDPNAG was used to activate bilirubin GT, it produced no increase in enzyme activity when employed with concentrations of BSA below 3–3.5 mg/ml. However, introduction of BSA at or above 4 mg/ml resulted in GT activation by UDPNAG (Fig. 5). If the activated GT activity was expressed as a percentage of the "native" value, UDPNAG enhanced enzyme activity in a fashion linearly related to the BSA concentration. Thus, with 4, 6, and 12 mg/ml BSA, UDPNAG enhanced GT activity by 26, 44, and 86%, respectively.

All of these effects of BSA on "native," and detergent- and UDPNAG-activated bilirubin GT were fully reproduced when microsomal fractions were prepared and resuspended in 150 m*M* KCl. Under comparable enzyme incubation conditions, however, the bilirubin GT activity in microsomes isolated in KCl was always lower (20–30%) than that observed in microsomes prepared in sucrose–EDTA.

Finally, when unconjugated bilirubin (0.214 m*M*) was dissolved in DMSO and no BSA was included in the enzyme incubation mixture, the activation profile of GT differed significantly from that observed in the presence of BSA. Thus, although "native" GT values in this situation approximated those seen with 6 mg/ml BSA (4.05 nmole/10 min/mg), no activation was produced by UDPNAG (Fig. 6). Detergents, including deoxycholate, digitonin and Triton X-100, enhanced GT activity (Fig. 6), but the optimal activating effect (50–100%) was far lower than that observed when bilirubin was dissolved in BSA (300–500%) (Fig. 4).

To determine whether these effects of BSA were due to technical artifacts during the extraction and/or diazotization procedure,

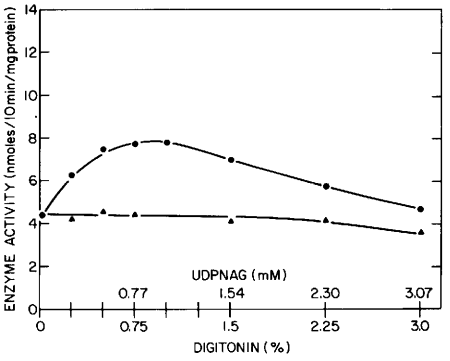

FIG. 6. Effect of UDPNAG (▲) or digitonin (●) on bilirubin GT activity when DMSO (2.5 *M*) was included in the enzyme incubation mixture. No albumin was present.

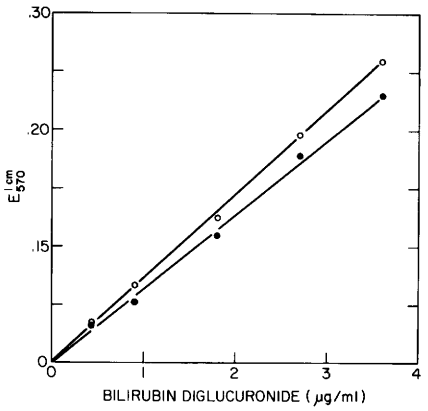

FIG. 7. Relationship between the concentration of sulfanilic acid azopigments derived from bilirubin diglucuronide and their extinction of 570 nm in presence of 2 (O) and 18 (●) mg/ml bovine serum albumin.

known amounts of bilirubin diglucuronide were extracted and diazotized (see Methods) in the presence of 2 or 18 mg/ml BSA. Under these conditions, formation of sulfanilic acid azopigment of bilirubin diglucuronide was essentially the same in the presence of either BSA concentration (Fig. 7). The E_{570} values with 2 and 18 mg/ml BSA were, respectively, 38.1 and 37.2 $\times 10^3$ M^{-1}, cm^{-1}.

Effect of rat albumin on bilirubin GT activity. When rat albumin was substituted for BSA, only part of the previously described effects were observed. "Native" bilirubin GT activity was similarly influenced by the presence of rat albumin and, as observed with BSA, the highest GT activity was observed with the lowest albumin concentration (2 mg/ml). However, GT activity of untreated microsomes in the presence of 2 mg/ml rat albumin accounted for only 60–70% of that found with similar concentrations of BSA, and could be enhanced by both detergents and UDPNAG. Thus, as illustrated in Fig. 8 and Table I, treatment of microsomes with deoxycholate resulted in GT activation, which was essentially the same when 2 or 6 mg/ml rat albumin were included. With 6 mg/ml, however, deoxycholate enhanced the "native" GT activity by 330–400% when BSA was used and 230–300% in the presence of rat albumin. Similarly, UDPNAG produced the same degree of activation in the presence of 2 or 6 mg/ml rat albumin (Fig. 9 and Table I). Dialysis did not alter the observed effects of rat albumin.

Influence of free bilirubin concentration on bilirubin GT activity. Calculated unbound bilirubin concentrations in solutions containing 0.214 mM bilirubin and 2–30 mg bovine or rat serum albumin (bilirubin:albumin molar ratio 0.49–7.3) are illustrated in Fig. 10. Although, in general, GT activity increases as the free bilirubin concentration increases, there is no simple relation between the free bilirubin concentration and measured values for GT. Moreover, at any given bilirubin:albumin molar ratio, GT activity of native microsomes is greater in the presence of bovine than rat albumin, although the estimated free bilirubin concentration is lower with bovine-derived proteins.

Effect of β-lactoglobulin on bilirubin GT activity. Replacement of albumin with β-lactoglobulin failed to reproduce the typical effects of BSA or rat albumin on bilirubin conjugation. "Native" GT activity (9–11 nmol/10 min/mg) was similar when 2 or 6 mg/ml of β-lactoglobulin were included and roughly resembled that obtained with 2 mg/ml BSA. Furthermore, GT activity in untreated microsomes could not be enhanced by either detergents or UDPNAG, regardless of the concentration of β-lactoglobulin present. On the contrary, addition of either UDPNAG (1.54–6.14 mM) or deoxycholate (0.05–0.2%) invariably resulted in inhibition of GT activity (Table I).

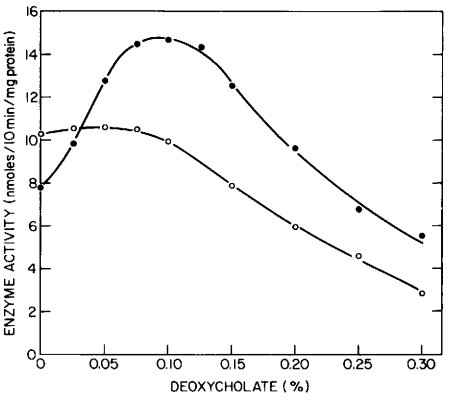

FIG. 8. Effect of deoxycholate on bilirubin GT activity in presence of 2 mg/ml bovine (O) or rat (●) serum albumin.

TABLE I. EFFECT OF BOVINE SERUM ALBUMIN, RAT ALBUMIN, AND β-LACTOGLOBULIN ON THE *IN VITRO* CONJUGATION OF BILIRUBIN BY RAT LIVER MICROSOMES

Enzyme preparation	Albumin (mg/ml)				β-Lactoglobulin (mg/ml)	
	Bovine		Rat			
	6	2	6	2	6	2
"Native"	100[a]	180	80	130	200	200
UDPNAG[b]	150	180	120	170	180	190
Deoxycholate[c]	350	170	240	270	160	170

[a] Values are percentages of the "native" enzyme activity obtained when bovine serum albumin was included in the enzyme incubation mixture of 6 mg/ml (88 μ*M*).
[b] Maximal activation obtained (usually observed with 3.07 m*M* UDPNAG).
[c] Maximal activation obtained (usually observed with 0.10% deoxycholate at these concentrations of albumins).

Lineweaver–Burk plot of "native" enzyme activity against UDP-glucuronic acid concentration in the presence of 6 mg/ml BSA or β-lactoglobulin (which resembled that obtained with 2 mg/ml BSA) revealed that Michaelis–Menten kinetics were followed in both of these situations. Similar apparent K_m values were obtained (5.43 and 5.68 m*M* with BSA and β-lactoglobulin, respectively) (Fig. 11).

Effect of BSA on p-nitrophenol GT activity. To determine whether these effects of BSA were also observed when another acceptor substrate of GT was employed, *p*-nitrophenol GT activity was measured in the presence of 2 and 12 mg/ml BSA. As illustrated in Fig. 12, both "native" and UDPNAG- or deoxycholate-activated enzyme activities were unaffected by different concentrations of BSA. As expected, the inhibitory effect of high concentrations of deoxycholate was buffered when 12 mg/ml BSA were included.

Discussion. The results of these studies have demonstrated that the presence of BSA in the reaction mixture profoundly influences the rate of *in vitro* conjugation of bilirubin by rat liver microsomes. Specifically, our studies have shown that when increasing concentra-

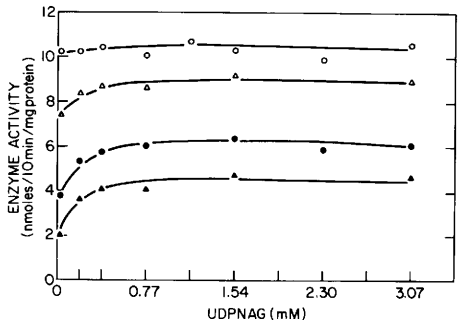

FIG. 9. Effect of UDPNAG on bilirubin GT activity in presence of bovine (circles) or rat (triangles) serum albumin at 2 (open symbols) and 6 (closed symbols) mg/ml.

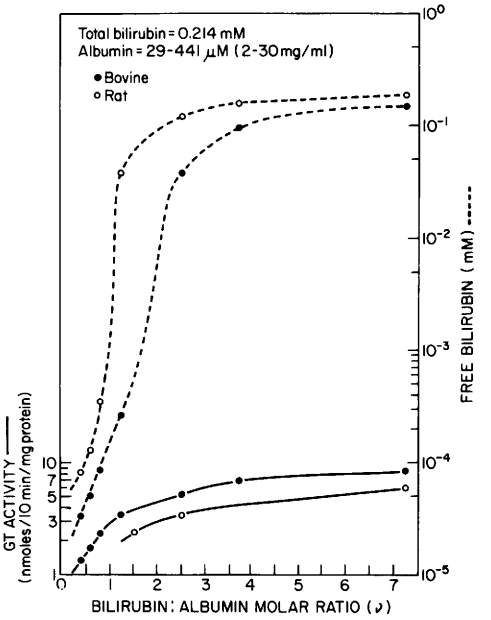

FIG. 10. Influence of bilirubin:albumin molar ratio (ν) on free bilirubin concentration and bilirubin GT activity in incubations containing 0.214 m*M* bilirubin and either bovine or rat albumin.

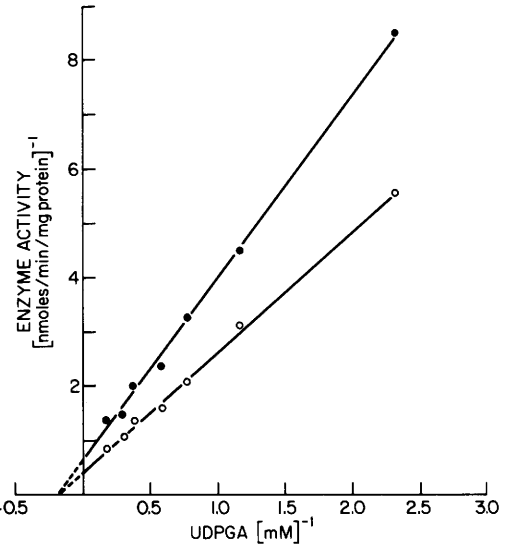

FIG. 11. Double-reciprocal plots of bilirubin GT activity against UDPGA concentration in presence of 6 mg/ml bovine serum albumin (●) or β-lactoglobulin (○).

tions of BSA are added to the incubation mixture proportionally higher concentrations of unconjugated bilirubin are necessary to produce optimal GT activity, and proportionally less conjugated bilirubin is formed when untreated microsomes are used. Higher concentrations of BSA require higher concentrations of detergents to produce maximal GT activation, whereas low concentrations (2 mg/ml) prevent enhancement of bilirubin conjugation by both detergents and UDPNAG. These effects were observed only in part when rat albumin was substituted for BSA and could not be reproduced when BSA was replaced by β-lactoglobulin. None of these effects of BSA on bilirubin conjugation could be demonstrated when *p*-nitrophenol was employed as the acceptor substrate for GT. Thus, it appears that BSA selectively interferes with the process of bilirubin conjugation *in vitro* by rat liver microsomes. Since in the liver cell, prior to its conjugation, bilirubin is bound to ligandin (24) rather than albumin, the relevance of these findings to its *in vivo* conjugation is unknown.

In attempting to explain these findings, an obvious hypothesis is that the effects on bilirubin GT activity of the various proteins studied are determined by their influence on the "free," or available substrate concentration. However, no obvious direct relationship between GT activity and the free bilirubin concentration was evident. Indeed, as noted above, at any molar ratio of bilirubin to albumin, the calculated free bilirubin concentration is lower with BSA than with rat albumin, although bilirubin GT activity is higher in the former case than the latter. Hence, the effects of different proteins in the incubation mixtures do not appear to be mediated solely by alterations in the free bilirubin concentration. This latter conclusion must be considered somewhat tentative, as the free bilirubin concentrations were calculated from binding constants highly dependent on methodology (16, 22, 25), using a simple mathematical model which ignores the binding of bilirubin to microsomes (26) and other trace constituents of the reaction mixture. A rigorous calculation of the free bilirubin concentration in the initial reaction mixture does not appear feasible at this time, both because of the complexities of the model, and the uncertainties in the various binding parameters.

That the precise basis for the observed effects of BSA remains obscure may reflect the complexity of the processes taking place in the microsomal reaction mixture, and the in-

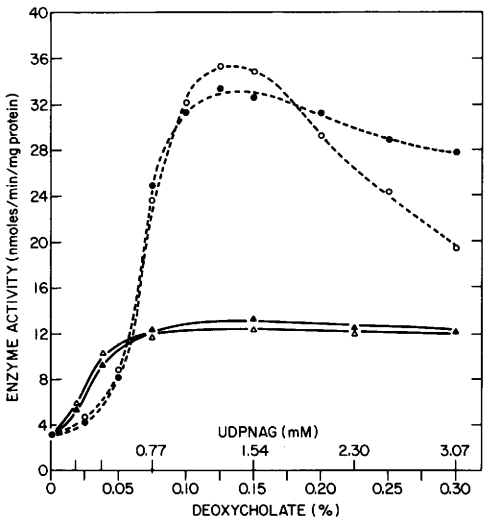

FIG. 12. Effect of UDPNAG (triangles) or deoxycholate (circles) on p-nitrophenol GT activity in presences of 2 (open symbols) and 12 (closed symbols) mg/ml bovine serum albumin.

adequacy of the relatively crude endpoint employed for their quantitation. Thus, bilirubin conjugation involves the formation of two isomeric (C_8, C_{12}) bilirubin monoglucuronides, as well as bilirubin diglucuronide, each of which may be formed at a different rate. Twice as much cosubstrate (UDPGA) is consumed per mole of bilirubin diglucuronide formed as per mole of bilirubin monoglucuronide, yet the extraction/diazotization procedure employed to quantitate conjugation does not distinguish between the formation of diconjugated and monoconjugated products.

Studies from our laboratory and others have demonstrated the formation of both bilirubin mono- and diglucuronides by native UDPNAG- and digitonin-activated rat liver microsomes (27). To accurately define the effect of BSA on these processes, the use of purified enzyme rather than microsomal preparations (9), and of high-performance liquid chromatography to determine the rates of formation of individual products (28) would be essential. While such studies are now becoming technically feasible in the basic biochemistry laboratory, they remain impractical in the setting of a clinical enzyme assay. Hence, studies of the sort described above will continue to be performed for some years to come, employing homogenates of microsomal preparations from very small samples of human liver and extraction and diazotization produces to estimate the overall rate of formation of conjugated products.

The present studies indicate that BSA profoundly affects the *in vitro* glucuronidation of bilirubin by rat liver microsomes, and, presumably by human liver when employed in analogous assay procedures. Because of the widespread use of BSA in *in vitro* measurements of bilirubin GT activity in human liver biopsy specimens, the importance of these results must be kept in mind. Caution will be necessary when comparing bilirubin GT activities from different laboratories, both with respect to its "native" and activated values, as different assay procedures may include different concentrations of BSA in the enzyme reaction mixture. Additionally, special consideration of the present findings is warranted in those studies which utilize DMSO to dissolve bilirubin (29), as the bilirubin GT activities, at least following activation by detergents or UDPNAG, may provide information considerably different from that obtained in the presence of albumin.

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1. Van Roy FP, Heirwegh KPM. Determination of bilirubin glucuronide and assay of glucuronyl transferase with bilirubin as acceptor. Biochem J **107**:507–518, 1968.
2. Strebel L, Odell GB. Bilirubin uridine diphosphoglucuronyl transferase in rat liver microsomes: Genetic variation and maturation. Pediatr Res **5**:548–559, 1971.
3. Isselbacher KJ, Chrabas MF, Quinn RC. The solubilization and partial purification of a glucuronyl transferase from rabbit liver microsomes. J Biol Chem **237**:3033–3036, 1962.
4. Dutton GJ. The biosynthesis of glucuronides. In: Dutton GJ, ed. Glucuronic Acid: Free and Combined. New York, Academic Press, p185, 1966.
5. Rao GS, Hauter G, Rao ML, Breuer H. An improved assay for steroid glucuronyl transferase in rat liver microsomes. Anal Biochem **74**:35–40, 1976.
6. Sanchez E, Tephly TR. Morphine metabolism. I. Evidence for separate enzymes in the glucuronidation of morphine and p-nitrophenol by rat hepatic microsomes. Drug Metab Dispos **2**:247–253, 1974.
7. Bock KW, von Clausbruch VC, Josting D, Ottenwalder H. Separation and partial purification of two differentially inducible UDP-glucuronyl transferases from rat liver. Biochem Pharmacol **26**:1097–1110, 1977.
8. Otani G, Abou-El-Makarem MM, Bock KW. UDP-glucuronyl-transferase in perfused rat liver and in microsomes. III. Effects of galactosamine and carbon tetrachloride on the glucuronidation of 1-naphtol and bilirubin. Biochem Pharmacol **25**:1293–1297, 1976.
9. Burchell B. Bilirubin UDP-glucuronyl transferase. In: Jacoby WB, ed. Methods in Enzymology. New York, Academic Press, p188, 1981.
10. Black M, Billing BH, Heirwegh KPM. Determination of bilirubin UDP-glucuronyltransferase activity in needle-biopsy specimens of human liver. Clin Chim Acta **29**:27–35, 1970.
11. Black M, Billing BH. Hepatic bilirubin UDP-glucuronyl transferase activity in liver disease and Gilbert's syndrome. N Engl J Med **280**:1266–1271, 1969.
12. Felsher BF, Carpio NM. Chronic persistent hepatitis

and unconjugated hyperbilirubinemia. Gastroenterology **76**:248–252, 1979.
13. Tavoloni N, Jones MJT, Wittman R, Kiang C-L, Berk PD. Comparison of different activators and diazotization procedures in the assay of bilirubin-UDP-glucuronyl transferase activity in rat liver. Clin Chim Acta **128**:209–221, 1983.
14. Brodersen R, Theilgaard J. Bilirubin colloid formation in neutral aqueous solution. Scand J Clin Lab Invest **24**:395–398, 1969.
15. Lee K, Gartner LM. Spectrophotometric characteristics of bilirubin. Pediatr Res **10**:782–788, 1976.
16. McDonagh AF. Bile pigments: Bilatrienes and 5,15-bilatrienes. In: Dolphin D, ed. The Porphyrins. New York, Academic Press, Vol 6:p293, 1979.
17. Berk PD, Kiang C-L, Stremmel W, Tavoloni N. A simple procedure for the isolation of bilirubin monoglucuronide and diglucuronide from bile. J Lab Clin Med **99**:559–547, 1982.
18. Tavoloni N, Jones MJT, Berk PD. Effect of low-dose phenobarbital on hepatic microsomal UDP-glucuronyl transferase activity. Biochem Pharmacol **32**:2143–2147, 1983.
19. Lowry OH, Rosebrogh NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
20. Weber AP, Schalm L. Quantitative separation and determination of bilirubin and conjugated bilirubin in human serum. Clin Chim Acta **7**:805–810, 1962.
21. Wolkoff AW, Ketley JN, Waggoner JG, Berk PD, Jacoby W. Hepatic accumulation and intracellular binding of conjugated bilirubin. J Clin Invest **61**:142–149, 1978.
22. Chen RF. The fluorescence of bilirubin–albumin complexes. In: Thaer AA, Sernetz M, eds. Fluorescence Techniques in Cell Biology. New York, Springer, p273, 1973.
23. Wosilait WD, Nagy P. A method of computing drug distribution in plasma using stepwise association constants: Clofibrate acid as an illustrative example. Comput Programs Biomed **6**:142–148, 1976.
24. Levi AJ, Gatmaitan Z, Arias IM. Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulphobromophthalein, and other anions. J Clin Invest **48**:2156–2167, 1969.
25. Lee K, Gartner LM. Bilirubin binding by plasma proteins: A critical evaluation of methods and clinical implications. Rev Perinat Med **2**:319–343, 1978.
26. Vessey DA, Goldenberg J, Zakim D. Differentiation of homologous forms of hepatic microsomal UDP-glucuronyl transferase. II. Characterization of the bilirubin conjugating form. Biochim Biophys Acta **309**:75–82, 1973.
27. Blanckaert N, Gollan J, Schmid R. Bilirubin diglucuronide synthesis by a UDP-glucuronic acid-dependent enzyme system in rat liver microsomes. Proc Natl Acad Sci USA **76**:2037–2041, 1979.
28. Gordon ER, Goresky CA. A rapid and quantitative high performance liquid chromatographic method for assaying bilirubin and its conjugates in bile. Canad J Biochem **60**:1050–1057, 1982.
29. Odell GB, Cuckier JO, Gourley GR. The presence of microsomal UDP-glucuronyl transferase for bilirubin in homozygous jaundiced Gunn rats and in the Crigler–Najjar syndrome. Hepatology **2**:307–315, 1981.

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