The Formation of Bilirubin and *p*-Nitrophenyl Glucuronides by Rabbit Liver

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1. Glucuronide formation of bilirubin and *p*-nitrophenol *in vitro* with excess of UDP-glucuronic acid by UDP-glucuronyltransferase from livers of young and adult rabbits was studied. 2. The development of UDP-glucuronyltransferase for the two substrates followed a markedly different pattern during maturation of young rabbits, *p*-nitrophenol-conjugation ability being much higher at birth than that for bilirubin. 3. Mg^{2+} increased bilirubin conjugation, but inhibited *p*-nitrophenyl glucuronide formation. 4. *p*-Nitrophenol acted as a potent non-competitive inhibitor for bilirubin conjugation but bilirubin did not affect *p*-nitrophenyl glucuronidation. 5. The enzyme for bilirubin conjugation was inactivated at pH 9 during treatment with snake venom, whereas in the same preparation the activity of the corresponding enzyme for *p*-nitrophenol was enhanced. In addition, some solubilization of the latter enzyme could be achieved by this method. 6. The possibility of the existence of more than one enzyme system for the formation of *O*-glucuronides is discussed.

The number of enzyme systems capable of *O*-glucuronide formation in mammalian liver remains uncertain. A definitive study of the microsomal enzyme, UDP-glucuronyltransferase, which is known to participate in this reaction, is difficult because the enzyme is insoluble. In addition, published reports cannot be compared, since enzyme preparations have been obtained from several animal species, and activity has been assayed with several aglycones, as well as bilirubin, a natural substrate for the enzyme. These non-biological substrates have been chosen since the use of bilirubin poses problems because of its insolubility, toxicity and light-sensitivity.

Early work (Axelrod, Schmid & Hammaker, 1957; Brown & Zuelzer, 1958) as well as the later experiments of Isselbacher, Chrabas & Quinn (1962) supported the concept of a single enzyme, but more recently a number of authors (Chojecki & Kern, 1961; van Leusden, Bakkeren, Zilliken & Stolte, 1962; Dutton, Langelaan & Ross, 1964; Storey, 1965*a,b*) have reported results indicating that there may be not one, but several, enzymes for *O*-glucuronide formation, each with unique substrate specificity. In these later experiments, significant variations were found in the conjugating abilities of liver preparations from different species of

animals, depending on which substrate and what conditions were used. All these experiments (except those of Isselbacher *et al.* 1962) were done with particulate preparations. The necessity of working with an insoluble enzyme source and the frequent choice of an unnatural substrate has obvious and recognized drawbacks (Dutton, 1963).

Since evidence for the formation of *O*-glucuronide with foreign substrates has accumulated, it seemed worth while to investigate the conjugating enzyme by using bilirubin, a more refractory but biologically important substrate. Our results provide a comparison of the biochemical properties and relative activities of UDP-glucuronyltransferase for the conjugation of bilirubin and *p*-nitrophenol obtained from rabbit liver during several stages of postnatal development.

EXPERIMENTAL

Materials and reagents

Crystalline bilirubin (Sigma Chemical Co., St Louis, Mo., U.S.A.) was dissolved in chloroform and extracted immediately before use with albumin at pH 9.5 according to the method of Grodsky & Carbone (1957). After extraction, the pH was adjusted to 7.4 with *N*-HCl. UDP-glucuronic acid, *p*-nitrophenol, aniline, lecithinase C, *Trimeresurus flavoviridis* venom and anthranilic acid were obtained from Sigma Chemical Co. Novobiocin was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.

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Analytical methods

With bilirubin as substrate. Bilirubin was protected from light throughout the procedure. The assays were performed in 10 ml. Erlenmeyer flasks capped with Parafilm. The incubation mix (Werder & Yaffe, 1964) consisted of 30 μ -moles of $MgCl_2$, 200 μ -moles of potassium phosphate buffer, pH 7.4, 0.2 μ -mole of UDP-glucuronic acid and approx. 0.40 ml. of enzyme preparation brought to a total volume of 3.5 ml. with 0.5% KCl. For inhibition experiments the UDP-glucuronic acid was increased to 0.8 μ -mole/flask. This concentration did not inhibit bilirubin conjugation. Control flasks did not contain UDP-glucuronic acid (since it was determined that this omission did not affect the result) and were incubated at 4°. The test flasks were incubated at 37° for 20 min. in a Dubnoff metabolic shaker (120 strokes/min.).

At the end of the incubation period, the flasks were chilled on ice and two 1.3 ml. portions removed from each flask. One portion was added to 0.7 ml. of diazo reagent (Grodsky & Carbone, 1957) and the second to 0.7 ml. of 0.18 N-HCl. From this point on, the procedure of Lathe & Walker (1958) was followed. Direct-reacting bilirubin was measured at 540 m μ on a Beckman model DU spectrophotometer. The total amount of bilirubin added to the reaction mixture was determined in each experiment (Malloy & Evelyn, 1937), and varied by $\pm 10\%$ from extract to extract.

With p-nitrophenol as substrate. p-Nitrophenol conjugation was measured by the method of Isselbacher *et al.* (1962), except that sodium phosphate buffer was used in place of the tris buffer. After the reaction was stopped with ethanol, the suspension was centrifuged, and a portion of the supernatant solution was diluted appropriately (usually 1:10) before the determination of $E_{400\text{ m}\mu}$. For some experiments, conjugation was determined in a reaction mixture similar to that used for bilirubin assays, with p-nitrophenol at concentrations ranging from 0.5 mM to 5 μ M. Bilirubin (0.05 mM) did not interfere with absorption by p-nitrophenol, provided that the concentration of the latter was greater than 0.1 mM. However, when the concentration of p-nitrophenol was lowered to 10 mM or to 1 mM, it was necessary to keep the assays, appropriately diluted, in 0.1 N-NaOH overnight. This treatment destroyed absorption at 400 m μ by bilirubin.

Protein assays were done by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Sigma Chemical Co.) as the standard.

Enzyme kinetics

It was established, with bilirubin as the substrate, that initial velocities were still found after a 5 min. incubation period. When inhibitors were used, bilirubin and the inhibitor were preincubated with the enzyme (in the absence of UDP-glucuronic acid) for 10 min. at 4° before the 5 min. incubation at 37°. Novobiocin was dissolved in water before use.

Preparation of enzyme

New Zealand rabbits were used throughout the study. Males were selected as soon as the animals were mature enough for the sex to be determined; otherwise animals were chosen randomly. When newborn or young rabbits were employed, the animals were supplied on the day of the experiment directly from the breeder. Older animals were

maintained on a diet of Purina Chow and water *ad libitum*. Adults were killed by the intravenous injection of air into the ear, whereas young rabbits were decapitated. After removal of the gall bladders, the livers were rinsed immediately with cold 0.1 M-KCl and homogenized with 3 vol. of the KCl for 2 min. in the cold. A Waring Blendor was used for the preparation of homogenates from adult rabbits and a glass homogenizer with a Teflon pestle for those from young animals. All subsequent procedures were carried out in the cold.

Solubilization

The methods of Isselbacher *et al.* (1962) and Pogell & Leloir (1961) were followed. In addition, the microsomes were subjected to the following procedures: (1) ultrasonic oscillation for 10 min. in a 9 kcyc./sec. Raytheon ultrasonic oscillator; (2) ultrasonic oscillation followed by incubation with digitonin as used by Pogell & Leloir (1961); (3) incubation for 1 hr. at 4° with 1.5 vol. of 2% (w/v) deoxycholic acid solution neutralized to pH 7.4; (4) ultrasonic oscillation as in (1) followed by incubation with deoxycholate as in (3); (5) incubation with stirring at 4° with 1.5 vol. of a solution of digitonin plus 1 mg. of ribonuclease/ml. for $\frac{1}{2}$ hr. or for 1 hr.; (6) incubation at 4° with 0.1 vol. of a 1% solution of lecithinase C overnight at pH 7.5; (7) addition of butan-1-ol to give a final concentration of 5% (v/v) over a 25 min. period with stirring, followed by incubation for 1 hr. at 4°.

In all cases, assays were performed on the microsomal suspension after treatment with the solubilizing agents before and after centrifugation at 105000g for 2 hr. in a Spinco model L ultracentrifuge with a no. 40 rotor.

RESULTS

Solubilization experiments

None of the methods employed for the solubilization of bilirubin-conjugating activity was successful. Either the enzyme was inactivated by the treatment (snake venom, deoxycholate, ribonuclease plus digitonin, lecithinase C, butanol), or no activity was found in the high-speed supernatant (ultrasonic oscillation, digitonin). An investigation of the effects of maintaining the microsomes at pH 9 overnight, which is a prerequisite for treatment with snake venom, showed that most of the enzyme activity was irreversibly lost as a result of the pH change alone, and a similar experiment at pH 8 gave a 50% loss of the conjugating ability in all instances. The microsomes were readjusted to pH 7.4 before the assay of enzyme activity. The addition of mercaptoethanol (0.2%) to the snake-venom incubation mixture did not prevent the inactivation of the enzyme at pH 9.

With p-nitrophenol as the substrate, not only was some solubilization of the conjugating enzyme obtained when snake venom was used, but in addition assays on the reaction mixture after treatment with snake venom, but before high-speed centrifugation, showed that enzyme activity was enhanced. Table 1 gives a comparison of assays

Table 1. *Solubilization of UDP-glucuronyltransferase by treatment with snake venom*

Specific activities are expressed as μ moles conjugated/mg. of protein in 20 min. at 37°. Units are expressed as total μ moles conjugated in 20 min. at 37°. Percentages solubilized refer to units as percentages of total μ moles conjugated by untreated microsomes in 20 min. at 37°.

	Untreated microsomes		Snake-venom mix		Supernatant		Pellet	
	<i>p</i> -Nitrophenol	Bilirubin	<i>p</i> -Nitrophenol	Bilirubin	<i>p</i> -Nitrophenol	Bilirubin	<i>p</i> -Nitrophenol	Bilirubin
Specific activity	37	1.63	143	0.53	33	0	155	0.49
Units	5320	236	14 508	54	1072	0	11 990	462
Percentage solubilized	(100)	(100)	279	23	21	0	229	20

done for a typical experiment with both substrates. In this experiment, *p*-nitrophenol conjugation was increased nearly threefold in the snake-venom mixture, whereas bilirubin conjugation was greatly decreased by the procedure. Assays on the supernatant from high-speed centrifugation showed that 21% of the conjugating activity for *p*-nitrophenol was solubilized by this procedure, but no measurable amount of bilirubin conjugation was found. Tripling the amount of snake venom increased solubilization of the *p*-nitrophenol enzyme to nearly 40%. The percentages are based on the conjugating ability of the original microsomal fraction.

Enzyme kinetics

Kinetic experiments were done with microsomes from adult animals as the source of the enzyme, and bilirubin as the substrate.

Effect of substrate concentration. Bilirubin in excess of 0.05 mM resulted in substrate inhibition when incubation was carried on for 20 min. At 0.075 mM-bilirubin there was a 25% decrease in conjugation, and at 0.1 mM a 40% decrease. Doubling the Mg^{2+} concentration did not reverse the inhibition. UDP-glucuronic acid (0.2 μ mole/flask) was in excess.

Effect of Mg^{2+} . The concentration of Mg^{2+} (8.6 mM) used in the bilirubin assays, which was optimum for conjugation of this substrate, inhibited *p*-nitrophenol conjugation. Fig. 1 shows the effects of Mg^{2+} on the conjugation of both substrates. Mg^{2+} at 10 mM decreased *p*-nitrophenol conjugation by 43%, whereas the elimination of Mg^{2+} from the reaction mixture with bilirubin as the substrate resulted in a 45% decrease in activity. Similar results were obtained with tris or phosphate buffer.

Effect of pH. The optimum pH for bilirubin conjugation was 7.4. There was no difference in activity with tris or phosphate buffer. A rapid decrease in bilirubin glucuronide formation occurred at pH values above 7.6, probably owing in part to hydrolysis of the ester bond by the alkali. However, as

Fig. 1. Effect of Mg^{2+} concentration on glucuronide formation of bilirubin and *p*-nitrophenol by rabbit-liver microsomes after 20 min. incubation at 37°. ●, Bilirubin; ▲, *p*-nitrophenol.

noted above, the enzyme itself is irreversibly inactivated by prolonged incubation at pH 8 and above.

Effect of inhibitors. As reported by Hargreaves & Holton (1962), novobiocin inhibited bilirubin conjugation. Kinetic experiments with the antibiotic at a concentration of 0.15 mM gave results indicative of non-competitive inhibition. Fig. 2 shows a Lineweaver & Burk (1934) plot of data from a typical experiment.

p-Nitrophenol also inhibited bilirubin glucuronide formation. At concentrations of 0.5 mM- and 50 μ M-bilirubin, 50–75% inhibition was observed, depending on the enzyme preparation. Again, kinetic experiments showed the effect to be non-competitive (Fig. 3). When the concentration of bilirubin was kept at 50 μ M and the influence of *p*-nitrophenol at 5 μ M and 10 μ M was examined, there was 22 and 35% inhibition of bilirubin conjugation

Fig. 2. Lineweaver-Burk plot showing inhibition by novobiocin (0.15 mM) of bilirubin glucuronide formation after 5 min. incubation at 37°. ○, No inhibitor; ●, novobiocin added.

Fig. 3. Lineweaver-Burk plot showing inhibition by *p*-nitrophenol (0.5 mM) of bilirubin glucuronide formation after 5 min. incubation at 37°. ○, No inhibitor; ●, *p*-nitrophenol added.

respectively. There was no decrease in *p*-nitrophenyl glucuronide formation in the presence of bilirubin, even when the latter was present in a tenfold excess.

Anthranilic acid, on the other hand, proved (at a concentration of 3 mM) to be a competitive inhibitor (30–40% inhibition) of bilirubin glucuronide formation (Fig. 4). *o*-Aminophenol, which is readily oxidized, and is therefore usually assayed in the presence of ascorbic acid (which inhibits the diazo reaction) (Lathe & Walker, 1958) at a concentration of 1 mM, gave no reduction of bilirubin conjugation in the absence of the antioxidant.

Developmental aspects of p-nitrophenol and bilirubin conjugation

Fig. 5 shows graphically the conjugating activities, as percentages of the adult male activity

Fig. 4. Lineweaver-Burk plot showing inhibition by anthranilic acid (3 mM) of bilirubin glucuronide formation after 5 min. incubation at 37°. ○, No inhibitor; ●, anthranilic acid added.

Fig. 5. UDP-glucuronyltransferase activity for bilirubin and *p*-nitrophenol in liver homogenates of rabbits of different ages. Assay mixtures were incubated at 37° for 20 min. ▲, Bilirubin; ●, *p*-nitrophenol.

(based on the conjugation/mg. of protein), for *p*-nitrophenol and bilirubin, with liver homogenates as the source of enzyme. Each point is the average of at least four separate experiments. The enzyme activity at any given age was reproducible to within $\pm 8\%$. It is evident that the development of the two enzymes does not follow the same course, activity with *p*-nitrophenol being much higher up to 4 days after birth than is bilirubin conjugation.

Inhibition studies with microsomes from 4-, 7- and 10-day-old rabbits with *p*-nitrophenol at

0.5 mM gave 88, 87 and 81% decreases in bilirubin conjugation, higher amounts than normally obtained with adult rabbits.

DISCUSSION

The results given in this paper suggest that bilirubin and *p*-nitrophenol may be conjugated by two different UDP-glucuronyltransferases in rabbit liver. There are four lines of evidence: developmental aspects, metal aspects, inhibition and solubilization experiments.

The development of the enzyme activity towards *p*-nitrophenol follows a markedly different pattern to that for bilirubin in the young rabbit. *p*-Nitrophenol conjugation is much higher at birth and reaches the adult activity faster than does bilirubin conjugation. Similar results for the formation of *p*-nitrophenyl and *o*-aminophenyl glucuronides in the perinatal mouse have been noted by Dutton *et al.* (1964) with *o*-aminophenol-conjugating ability, as is the case for bilirubin, developing more slowly than that for *p*-nitrophenol. However, the same authors found that transferase activity was higher towards *p*-nitrophenol than *o*-aminophenol in the foetal rat but not in the neonatal rat. Lack of parallelism between rates of synthesis of bilirubin and *o*-aminophenyl glucuronides in both tissue slices and suspensions of livers from a number of animals was reported by Lathe & Walker (1958), and, moreover, bilirubin, but not *o*-aminophenol, was glucuronidated by liver slices from the cat. Another aspect of the situation has been investigated by van Leusden *et al.* (1962), who studied *p*-nitrophenol and bilirubin conjugation in livers of adult homozygous Gunn rats. These animals, which are jaundiced, presumably because of low activity of the enzyme necessary for bilirubin glucuronide formation, nevertheless possess the ability to conjugate *p*-nitrophenol.

Bilirubin glucuronide formation in the rabbit-liver microsomes was shown to be stimulated by the presence of Mg^{2+} , whereas, under the same circumstances, *p*-nitrophenol conjugation was inhibited. Dutton & Storey (1954) and Storey (1965b), using mouse-liver homogenates, reported a similar increase in *o*-aminophenol conjugation and decrease in *p*-nitrophenol conjugation in the presence of Mg^{2+} . The inhibition of *p*-nitrophenyl glucuronide formation by Mg^{2+} , as well as by Ca^{2+} , was also found by Isselbacher *et al.* (1962) with a solubilized partially purified enzyme preparation from rabbit liver. This agreement of results indicated that the differences in response to metal ions for the three substrates is probably not merely one of permeability (which can be a factor when particulate enzyme preparations are used) but could be a manifestation of the presence of several enzymes

rather than one. Alternatively, the ions may influence active-centre interactions for different substrates on the same enzyme.

The results obtained from snake-venom solubilization experiments with *p*-nitrophenol and bilirubin as substrates, i.e. a 300% enhancement of *p*-nitrophenol conjugation coupled with an 80% loss in bilirubin glucuronidation, also support the concept of two enzymes rather than one. Whether the glucuronyltransferase for bilirubin was, in fact, partially solubilized by the treatment could not be determined, since enzyme activity was so low after incubation with the snake venom. Failure of this and the other methods to solubilize bilirubin-conjugating activity (especially the repeated inactivation by treatments with lecithinase C, *n*-butanol or deoxycholate) could indicate the destruction, by esterases in the snake venom for example, of a lipid factor similar to that reported by Imai & Sato (1960) for aniline hydroxylase.

Kinetic data lend further credence to the existence of more than one enzyme system. *p*-Nitrophenol was a potent inhibitor of bilirubin glucuronidation, giving nearly 25% inhibition at 5 mM, but the effect was non-competitive. If the same enzyme conjugated both substrates, one would expect a competitive type of inhibition to occur. On the other hand, in the same system bilirubin, even at a ten-fold excess, was unable to decrease *p*-nitrophenol conjugation. Inhibition studies with *p*-nitrophenol and *o*-aminophenol by Storey (1965b) showed *p*-nitrophenol to be a competitive inhibitor of *o*-aminophenol conjugation, whereas *o*-aminophenol was a less effective and non-competitive inhibitor for *p*-nitrophenol. Novobiocin acted similarly with bilirubin as with *p*-nitrophenol, *o*-aminophenol and 4-methylumbelliferone (Lokietz, Dowben & Hsia, 1963), giving non-competitive inhibition with all of these substrates.

Despite similarities in the behaviour of the conjugating enzyme(s) for bilirubin and *o*-aminophenol (Mg^{2+} requirement, developmental aspects), no inhibition of bilirubin conjugation by *o*-aminophenol was found, even at 100-fold excess of the latter. However, such an assay is difficult to perform because of the rapid oxidation of *o*-aminophenol in the absence of an antioxidant such as ascorbic acid, whereas this compound inhibits the diazo reaction (Lathe & Walker, 1958) for the measurement of bilirubin glucuronide. Anthranilic acid, on the other hand, which like bilirubin forms an ester glucuronide, inhibited bilirubin conjugation competitively, a large excess (100-fold) of the inhibitor being required. Isselbacher *et al.* (1962), using an enzyme purified from rat liver, reported similar results for inhibition of *p*-nitrophenol conjugation by anthranilate, but Lathe & Walker (1958) were unable to inhibit bilirubin conjugation

in rat-liver slices with this compound; however, only a sevenfold excess of the anthranilate was used. It is difficult to explain how anthranilic acid can inhibit competitively the formation of both ether and ester glucuronides. One might visualize a multi-stage reaction, with anthranilic acid competing with bilirubin and *p*-nitrophenol at different stages. This explanation could also be used for the anomalous results of Storey (1965*b*) with *o*-aminophenol and *p*-nitrophenol.

Microsomal preparations have been used in this and in other Laboratories because of the inherent difficulty of obtaining UDP-glucuronyltransferase in a soluble form. Isselbacher *et al.* (1962), using a preparation of *Trimeresurus flavoviridis* venom obtained from Japan, were able to solubilize and partially purify, with a threefold increase in activity, the enzyme that conjugates *p*-nitrophenol. The same purified preparation also conjugated *o*-aminophenol and anthranilic acid, and, to a lesser extent, bilirubin. The differences in solubilization between our results and those obtained by these authors may be due to variations in the snake venom used, since increasing the amount of venom in our experiments did increase the amount of solubilization of *p*-nitrophenol glucuronyltransferase. This report by Isselbacher *et al.* (1962) is the only published account of kinetic experiments with a soluble UDP-glucuronyltransferase.

Our conclusion, from the present results and from evidence presented by other Laboratories, is that there appear to be valid differences in the requirements and properties of UDP-glucuronyltransferase for different substrates, which may indicate the existence of not one non-specific enzyme, but of a family of enzymes each with its own substrate specificity.

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