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# BILIRUBIN AND THE HETEROGENEITY OF MICROSOMAL URIDINE DIPHOSPHATE GLUCURONYLTRANSFERASE FROM RAT LIVER

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SUMMARY

1. The glucuronide conjugation of bilirubin by rat liver microsomal UDP-glucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17) was studied; the enzyme preparation was activated by addition of Triton X-100.

2. When bilirubin was dissolved in NaOH solution or solubilized in an albumin solution, equal initial velocities could be obtained at pH 7.3, but much higher concentrations of albumin-solubilized bilirubin were required for this.

3. Bilirubin glucuronide conjugation could be inhibited by other substrates such as *p*-nitrophenol, 4-methylumbelliferone, phenolphthalein and *o*-aminophenol.

4. *p*-Nitrophenol glucuronide conjugation was inhibited by very low concentrations of bilirubin (10 $\mu$M) only if bilirubin was added in the absence of albumin. With increasing concentrations of albumin this inhibition disappeared. Increasing concentrations of microsomal enzyme protein also reversed the inhibitory effect of bilirubin. The inhibition appeared to be competitive.

5. Contrary to most earlier findings, the present results suggest that bilirubin and *p*-nitrophenol are conjugated at the same active site.

## INTRODUCTION

A great number of substances are conjugated *in vivo* with glucuronic acid by the enzyme UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17). In a previous publication[1] it was reported that *p*-nitrophenol, phenolphthalein, 4-methylumbelliferone and *o*-aminophenol appear to be conjugated *in vitro* at the same active site by one enzyme. With all of these substrates $\beta$-D-glucuronides are formed by way of an ether bond at a phenolic hydroxyl group on the substrate molecules. These are, however, artificial substrates and it would be important to find out, whether bilirubin, which is a physiological substrate for UDPglucuronyltransferase, is also conjugated at the same active site. The glucu-

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ronide conjugation of bilirubin is difficult to assay *in vitro*, particularly because it is virtually insoluble in water[2]. This has recently been discussed[3]. The most frequently used procedures[4–8] overcome this by solubilizing the bilirubin through binding to bovine or human serum albumin. As a consequence, the characteristics of this binding to albumin will influence the kinetics of the enzyme–substrate reaction, the measured kinetics being the resultant of the two. Adlard and Lathe[9] have suggested that this is the cause of anomalies they found in the kinetics of bilirubin conjugation. Moreover, the concentration of free, soluble bilirubin (which presumably is the substrate form) in the presence of bovine serum albumin will be only a fraction of the formal concentration due to the very high association constant of the bilirubin–albumin complex[10].

Several investigations have suggested that *p*-nitrophenol and bilirubin are conjugated by different enzymes, on the basis of kinetic observations[5,6] and on differences in response to ethanol treatment *in vivo* and partial hepatectomy[11]. Moreover, with *p*-nitrophenol a phenolic hydroxyl group is conjugated and with bilirubin a carboxyl group, resulting in "ether" and "ester" glucuronides, respectively. Further evidence concerning multiplicity of UDPglucuronyltransferase has been summarized by Dutton[12,13].

In this work evidence will be presented that bilirubin and *p*-nitrophenol are conjugated by the same active site and that high concentrations of protein, used to solubilize bilirubin, led to the suggestion of heterogeneity of UDPglucuronyltransferase towards *p*-nitrophenol and bilirubin in earlier kinetic studies[5,6].

## MATERIALS AND METHODS

Microsomes were prepared from the liver of male rats (Wistar, TNO, Zeist, The Netherlands, 200–300 g) essentially by the method of Hewick and Fouts[14]. Washed microsomal pellets were resuspended in 0.154 M KCl to give a protein concentration of about 11 mg/ml. The preparations were stored at −40 °C and suffered no loss of activity of UDPglucuronyltransferase for at least two months.

UDPglucuronyltransferase activity with *p*-nitrophenol, phenolphthalein, 4-methylumbelliferone and *o*-aminophenol as substrates was measured as described before[1] in 75 mM Tris–HCl, pH 7.3, which contained 5 mM $MgCl_2$. After being diluted to about 3 mg microsomal protein/ml, enzyme preparations were activated before use by addition of 0.25% (v/v) Triton X-100[15].

With bilirubin as substrate the method of Van Roy and Heirwegh[7] was followed. In some experiments the substrate bilirubin (E. Merck A.G. Darmstadt, Germany) was solubilized in bovine serum albumin, dialyzed against 1 mM EDTA, as described by them; the albumin concentration in the incubation medium was 2.9 mg/ml (about 40 $\mu$M). In other experiments bilirubin was dissolved in 0.015 M NaOH and used immediately after this in the incubation media; this did not affect the pH of the incubation medium. The buffer used was the same as that for *p*-nitrophenol conjugation. UDP-glucuronate (disodium salt; Boehringer Mannheim, Germany) was in both cases 1.5 mM in the incubation medium. With bilirubin as substrate also Triton X-100 (0.25%) was used to activate the enzyme activity optimally to about 300–400% of the original activity. Typical bilirubin conjugating activity was 0.4–0.6 nmole/min per mg microsomal protein in this system.

For *p*-nitrophenol as substrate 200 $\mu$g microsomal protein per ml of incubation

medium during incubations of 15 min was used; for bilirubin this was 2 mg/ml for incubations of 5–10 min.

Protein was determined by the method of Lowry *et al.*[16] as described by Layne[17] with bovine serum albumin (Poviet, Amsterdam, The Netherlands) as standard.

## RESULTS

### *Solubilization of the substrate*

A very low activity of UDPglucuronyltransferase was found by some investigators when an aqueous solution of bilirubin was used as substrate instead of albumin solubilized bilirubin[4,7]. However, as shown in Fig. 1 equal initial velocities for the enzyme reaction were observed in the present work but the velocity with alkaline-dissolved substrate decreased earlier in time than with albumin-solubilized substrate. In this experiment 30 $\mu$M bilirubin, applied in alkaline-dissolved form, was used as substrate concentration and 120 $\mu$M with albumin-solubilized bilirubin. With alkaline-dissolved bilirubin the same initial velocity as with 30 $\mu$M was found at 15 and 120 $\mu$M; so it seems that for this substrate form there is already substrate saturation at 15 $\mu$M. At bilirubin concentrations below 15 $\mu$M the measurements were no longer reliable, therefore in the present work no $K_m$ value from a Lineweaver–Burk plot could be obtained.

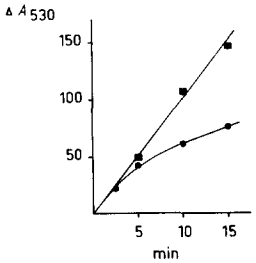



Fig. 1. Glucuronide conjugation of bilirubin measured as a function of time. Bilirubin was applied to the incubation medium either solubilized in bovine serum albumin (final concentration in the incubation medium about 40 $\mu$M) solution (120 $\mu$M, ■—■) or dissolved in 0.015 M NaOH (30 $\mu$M, ●—●). The velocity of the reaction is expressed as the absorbance at 530 nm during the incubation.

With albumin-solubilized bilirubin, on the other hand, the initial velocity at 30 $\mu$M is only about half of that at 120 $\mu$M at which concentration there is substrate saturation. Thus, a great difference of $K_m$ is suggested between the two substrate forms. Some experiments in which bilirubin-albumin ratios were varied at different bilirubin concentrations suggested that this ratio might strongly influence results of kinetic studies as suggested by Adlard and Lathe[9]. Therefore, kinetic experiments with albumin-solubilized bilirubin as substrate have not been performed in this study.

### *Inhibition of bilirubin conjugation*

The inhibition of bilirubin glucuronide conjugation by some other substrates is shown in Table I. Bilirubin solubilized in an albumin solution was used as substrate.

TABLE I

INHIBITION OF BILIRUBIN GLUCURONIDE CONJUGATION BY SOME OTHER SUBSTRATES OF UDP-GLUCURONYLTRANSFERASE

Bilirubin (120 $\mu$M) was added to the incubation medium solubilized in bovine serum albumin (final concentration about 40 $\mu$M); the enzyme preparation was a microsomal preparation. Phenolphthalein and 4-methylumbelliferone dissolved in 50% (v/v) ethanol–water were added to the incubation medium, resulting in 2.5% (v/v) ethanol in the incubation medium.

| *Inhibitor* | *Concn (mM)* | *% Inhibition* |
|---|---|---|
| p-Nitrophenol | 0.30 | 56 |
| | 1.20 | 87 |
| 4-Methylumbelliferone | 0.30 | 51 |
| | 1.20 | 93 |
| Phenolphthalein | 0.075 | 48 |
| | 0.150 | 77 |
| *o*-Aminophenol | 1.0 | 9 |
| | 2.0 | 19 |

None of the compounds tested influences the colour development with the ethylanthranilate reagent after the incubation. *p*-Nitrophenol and 4-methylumbelliferone showed equal inhibition but phenolphthalein was much stronger. *o*-Aminophenol at 2 mM was also inhibitory; in the experiments with this latter compound the concentration of ascorbic acid was reduced (from normally 200 $\mu$g per ml of incubation medium to 20 $\mu$g per ml) as its presence inhibits colour development with the ethyl anthranilate reagent.

With a postnuclear supernatant used in previous studies as enzyme preparation[1] about the same degrees of inhibition of bilirubin glucuronide conjugation, by the other substrates, were found.

*Bilirubin as inhibitor of p-nitrophenol glucuronidation*

When bilirubin, solubilized in bovine serum albumin, was tested for its ability to inhibit *p*-nitrophenol glucuronide conjugation, no inhibition was found. If, however, bilirubin was added without albumin but dissolved in an NaOH solution, considerable inhibition occurred. The presence of increasing concentrations of albumin appeared to reduce the degree of inhibition of *p*-nitrophenol glucuronide conjugation by bilirubin. Even the inhibitory effect of a bilirubin concentration as high as 240 $\mu$M (added to the incubation medium dissolved in 0.015 M NaOH), which more than saturates the incubation medium (pH 7.3), could be totally reversed by the addition of bovine serum albumin (Fig. 2). In this experiment it seems remarkable that a concentration of about 10 $\mu$M albumin (600 $\mu$g/ml) gives full protection against bilirubin. The explanation may be that bilirubin is colloidally bound by albumin[18]. The inhibitory effect of lower concentrations of bilirubin in the incubation medium could be reversed by lower concentrations of albumin.

As is to be expected, the concentration of protein from the microsomal enzyme preparation will determine the degree of inhibition by bilirubin of *p*-nitrophenol glucuronidation. Table II depicts an experiment in which the microsomal enzyme protein concentration and the incubation time were varied reciprocally, such that during the incubation the same amount of *p*-nitrophenol would be converted with all

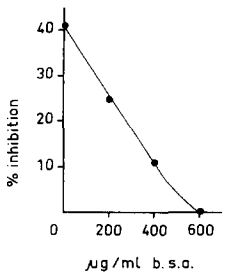



Fig. 2. Effect of bovine serum albumin (b.s.a.) on the degree of inhibition of *p*-nitrophenol glucuronide conjugation by bilirubin. The *p*-nitrophenol concentration was 0.30 mM and 240 μM bilirubin was used as inhibitor concentration. Bilirubin was dissolved in 0.015 M NaOH. The concentration of microsomal protein was 180 μg/ml of incubation medium.

TABLE II

EFFECT OF MICROSOMAL ENZYME PROTEIN CONCENTRATION ON INHIBITION BY BILIRUBIN OF *p*-NITROPHENOL GLUCURONIDATION

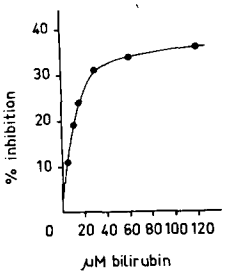
Different concentrations of microsomal enzyme protein were incubated in the presence or absence of 60 μM bilirubin dissolved in 0.015 M NaOH for different incubation times, such that the amount of substrate converted during the incubations without bilirubin present, was the same. The inhibition of *p*-nitrophenol glucuronidation (the substrate concentration being 0.30 mM) by bilirubin was measured.

| *μg microsomal protein per ml of incubation medium* | *Incubation time (min)* | *μmoles p-nitrophenol converted during the incubations without bilirubin present* | *% inhibition by bilirubin* |
|---|---|---|---|
| 42.5 | 60 | 0.11 | 63 |
| 85 | 30 | 0.14 | 52 |
| 170 | 15 | 0.14 | 36 |
| 340 | 7.5 | 0.14 | 19 |

of the enzyme concentrations tested. As shown, the most inhibitory effect was found at the lowest enzyme concentration. Thus, at high enzyme protein concentrations a lack of inhibition by bilirubin may be found. Without Triton X-100 activation, indeed, higher protein concentrations are required in the incubation mixture.

Furthermore, the enzyme activity towards *p*-nitrophenol appeared to be very sensitive to bilirubin in the absence of albumin as a concentration of 10 μM bilirubin was already very inhibitory (Fig. 3). The degree of inhibition decreased at higher *p*-nitrophenol concentrations. The increasing concentrations of bilirubin indicated in Fig. 3 are formal concentrations of which a steadily increasing part flocculates out of solution at pH 7.3. Thus, the effective inhibitor concentration may not vary linearly with the indicated concentration. This might be the reason why inhibition seems to level off at about 60 μM bilirubin.

Competitive inhibition of *p*-nitrophenol glucuronide conjugation by bilirubin was indicated by a Lineweaver–Burk plot, in which the concentration of *p*-nitrophenol was varied with or without 30 μM bilirubin (dissolved in NaOH) present (Fig. 4). In contrast with previous experiments in which a postnuclear supernatant was used



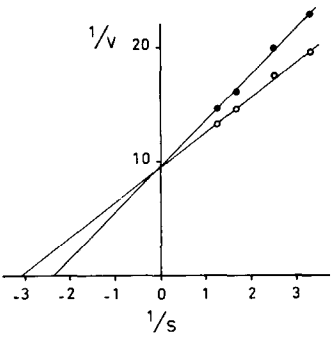




Fig. 3. Inhibition of *p*-nitrophenol glucuronide conjugation by bilirubin. Bilirubin was added dissolved in 0.015 M NaOH. The *p*-nitrophenol concentration was 0.30 mM during incubation of 15 min. The microsomal protein concentration was 220 μg/ml of incubation medium.

Fig. 4. Inhibition by bilirubin of *p*-nitrophenol glucuronide conjugation. Double-reciprocal plot of initial velocity; various *p*-nitrophenol concentrations in the absence (○—○) and presence (●—●) of 30 μM bilirubin dissolved in 0.015 M NaOH. Velocity is expressed as μmole of *p*-nitrophenol converted per min per mg microsomal protein.

as enzyme preparation[1], with microsomes a distinct substrate inhibition was found at 1.7 mM *p*-nitrophenol.

At bilirubin concentrations higher than 120 μM, still higher degrees of inhibition can be obtained; these, however, were not studied because, as suggested by Fig. 3, some other effect of bilirubin than substrate competition may play a role at those concentrations.

*Bilirubin as inhibitor of glucuronidation of some other substrates*

Alkaline-dissolved bilirubin was also inhibitory to 4-methylumbelliferone glucuronide conjugation (Table III). The degree of inhibition by bilirubin decreased at higher substrate concentration. With phenolphthalein as substrate only a very small

TABLE III

INHIBITION OF GLUCURONIDATION BY BILIRUBIN

Alkaline-dissolved bilirubin was added to incubation media in which glucuronide conjugation of 4-methylumbelliferone, phenolphthalein and *o*-aminophenol was measured. Microsomes activated by Triton X-100 were used as enzyme preparation. Phenolphthalein and 4-methylumbelliferone were added dissolved in aqueous ethanol solutions resulting in 2.5 and 2.0% (v/v) ethanol, respectively, in the incubation medium.

| *Substrate* | *Concn (mM)* | *Microsomal protein concn in incubation (μg/ml)* | *Bilirubin inhibitor concentration (μM)* | *% inhibition* |
|---|---|---|---|---|
| 4-Methylumbelliferone | 0.18 | 165 | 15 | 16 |
| | 0.18 | 165 | 60 | 22 |
| | 0.72 | 165 | 15 | 9 |
| | 0.72 | 165 | 60 | 15 |
| Phenolphthalein | 0.075 | 220 | 15 | 5 |
| | 0.075 | 220 | 60 | 7 |
| *o*-Aminophenol | 1.0 | 3000 | 15 | 14 |
| | 1.0 | 3000 | 60 | 25 |

inhibitory effect was found which is in agreement with previous findings[1] that much higher concentrations of other substrates, used as inhibitors, are needed for inhibition of phenolphthalein glucuronidation than for 4-methylumbelliferone as substrate.

*o*-Aminophenol glucuronidation was inhibited by bilirubin even at a relatively very high protein concentration (3000 $\mu$g/ml), required for a measurable *o*-aminophenol glucuronidation. The degree of inhibition was not dependent on the incubation time. Thus, this inhibition was different from that reported for the other substrates as inhibitors of *o*-aminophenol glucuronidation[1].

## DISCUSSION AND CONCLUSION

The present results, obtained with a detergent-activated microsomal enzyme preparation suggest that *p*-nitrophenol and bilirubin are conjugated with glucuronic acid at the same active site. This is based on the mutual inhibition of both substrates and the kinetic characteristics of the inhibition by bilirubin of *p*-nitrophenol glucuronide conjugation as shown in the Lineweaver–Burke plot (Fig. 4). The competitive characteristics of the inhibition by bilirubin of *p*-nitrophenol conjugation seem to exclude a nonspecific inhibition by bilirubin, due to some effect on membrane conformation.

This seems, however, to disagree with other findings. Tomlinson and Yaffe[5] reported non-competitive inhibition by *p*-nitrophenol of bilirubin glucuronide conjugation, whereas bilirubin could not inhibit *p*-nitrophenol conjugation. Halac and Reff[6] found that bilirubin glucuronide conjugation could be inhibited by phenolphthalein but not by *p*-nitrophenol. Conversely, bilirubin did not inhibit *p*-nitrophenol conjugation in their experiments.

In both of these studies albumin was used to solublize bilirubin and thus albumin concentrations higher than 1 mg/ml of incubation medium were obtained; total protein concentration was still higher due to protein from the enzyme preparation. If this is taken into consideration it seems quite obvious from the present results that bilirubin in their experiments[5,6] did not inhibit *p*-nitrophenol glucuronide conjugation. The protein concentration in the incubation medium may have been too high and, thus, bilirubin was no longer inhibitory.

The inhibition of bilirubin glucuronide conjugation by the other substrates confirms previous findings that phenolphthalein is much more inhibitory towards the conjugation of other substrates than *p*-nitrophenol and 4-methylumbelliferone, whereas the latter substrates are about equally inhibitory[1]. On the other hand, it is difficult to fit in the finding of Halac and Reff[6] that *p*-nitrophenol does not inhibit bilirubin glucuronide conjugation. Inhibition by *o*-aminophenol has been reported by others[4].

The characteristics of inhibition by alkaline-dissolved bilirubin of 4-methylumbelliferone glucuronide conjugation are in accord with the same active site converting both 4-methylumbelliferone and bilirubin as suggested previously by Frezza *et al.*[19]. Although with *o*-aminophenol as substrate the protein concentration was rather high (Table III) there is still inhibition of its glucuronidation by bilirubin. Previously[1] it has been shown that the conjugation of *o*-aminophenol was inhibited by *p*-nitrophenol, phenolphthalein and 4-methylumbelliferone such that first these inhibitors were glucuronidated during the incubation and only after conversion of these, *o*-aminophenol was glucuronidated. It was suggested that this might be compe-

titive inhibition with *o*-aminophenol in a very unfavourable position[1]. Apparently, the low concentration of truly dissolved bilirubin during the incubation for *o*-aminophenol glucuronidation is sufficient for the inhibition of this substrate.

If bilirubin and *p*-nitrophenol are converted at the same active site the $K_m$ for bilirubin as substrate should be the same as the $K_i$ for bilirubin as inhibitor of *p*-nitrophenol glucuronide conjugation. The present results suggest a great difference in $K_m$ between both bilirubin substrate forms in the present experiments and, thus, between the respective $K_i$ values. Therefore, a great difference in the degree of inhibition of *p*-nitrophenol glucuronidation by albumin-solubilized or alkaline-dissolved bilirubin is probably a result of a great difference in the $I/K_i$ ratio. Kinetic studies performed by others yielded $K_m$ values ranging from 10–300 $\mu$M for bilirubin, measured at several pH values and with bilirubin applied in several protein-bound or dissolved forms to the incubation medium[3,20,21]. Comparison of these values found in the literature did not show a clear difference between the published $K_m$ values for albumin-solubilized or alkaline-dissolved bilirubin.

A possible reason for the early decline in activity observed when alkaline-dissolved bilirubin is added to the incubation medium (Fig. 1) is that part of this bilirubin forms a colloidal solution in the incubation medium at pH 7.4, from which it is very difficult to release in a truly mono-molecularly dissolved form. During the conversion of all the truly dissolved bilirubin by enzyme activity there is no quick replenishment, thus the reaction velocity declines. With albumin-solubilized bilirubin this truly dissolved bilirubin remains in equilibrium with albumin-bound bilirubin and, therefore, remains at a constant concentration during the time observed.

Hakim *et al.*[11] suggested the existence of two distinct UDPglucuronyltransferases in rat liver for *p*-nitrophenol and bilirubin based on different responses of the enzyme activity towards both substrates on ethanol administration *in vivo* and partial hepatectomy. Their results are, in part, conflicting with other results[22]. However, as suggested previously[15] and as shown very clearly by Winsnes[23,24] and Lucier *et al.*[25] the results of this kind of experiment depend on whether non-activated enzyme preparations were used (as Hakim *et al.*[11] used) or activated preparations, and thus present no evidence on heterogeneity.

The Gunn rat seems to present the most serious argument for heterogeneity of UDPglucuronyltransferase. However, the effect of diethylnitrosamine on *o*-aminophenol glucuronide conjugation in the Gunn rat[26,27] indicates some differences in active site conformation between Gunn rats and other rat strains. The absence of bilirubin conjugating activity might have a similar cause. Thus, Gunn rats might not be deficient in a UDPglucuronyltransferase specific for bilirubin, but the changed UDPglucuronyltransferase which is found in the Gunn rat may not be able to bind bilirubin properly.

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