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# BILIRUBIN GLUCOSYL- AND GLUCURONYLTRANSFERASES

## A COMPARATIVE STUDY AND THE EFFECTS OF DRUGS

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**Abstract**—The hepatic enzymes that catalyze the conjugations of bilirubin with glucose and glucuronic acid were measured in the microsomal fractions of a few species of animals: rat, mouse, cat, guinea-pig, hamster and man. In all instances, the specific activities of the UDP-glucosyltransferase, when expressed as micrograms bilirubin "equivalents" conjugated per hour per milligram protein were lower than the corresponding values obtained for the UDP-glucuronyltransferase. The cat, which is peculiar in that it possesses low transglucuronidating activity, is able to conjugate bilirubin with glucose at a rate comparable to those of the other species of animals. A deficiency of both of these enzymes was demonstrated in the young rats; there was a gradual increase of both enzymes until about 50 per cent of adult value was reached in 4 weeks. The effects of phenobarbitone, and "13B" mixture (a Chinese drug) on the conjugating systems of bilirubin and harmol were also studied.

THE CONJUGATION of bilirubin with glucuronic acid is considered to be essential for its excretion.[1] An analogous reaction may occur with glucose as shown by the presence of bilirubin glucoside in dog bile,[2] and the formation of this conjugate has recently been accomplished using a rat liver microsomal preparation.[3] With the discovery of this novel biosynthetic pathway, it is interesting to compare the activities of these two glycosyltransferases in various animals and to evaluate their relative significance. Of particular interest in this respect is the cat which appeared to have low transglucuronidating activity when tested with exogenous aglycones;[4,5] it has also been shown to have a limited ability to form bilirubin glucuronide.[6] The high susceptibility of this animal to toxic agents could be attributed to its low activity of drug-metabolizing enzymes. However, it is plausible that it utilizes the alternative transglucosylating pathway for the transformation of bilirubin and for its subsequent excretion.

These two transglycosylation processes of bilirubin were also studied in young animals, where a deficiency of bilirubin glucuronyltransferase has been considered to be the primary cause of neonatal jaundice.[4,7,8] In this connexion, it was noted that among Singapore Chinese and Malay infants, there appeared to be a high incidence of severe neonatal jaundice of a non-hemolytic type.[9] The use of traditional Chinese herbs during pregnancy, especially the "13B" mixture has been thought to be a contributing factor. In addition, there seems to be an inherent transient hepatic dysfunction in Chinese infants in bilirubin conjugation in the first 2 weeks of life when compared to British infants.[10] The fact that phenobarbitone significantly reduced the bilirubin levels in Chinese newborn infants[11] seemed to support this postulation. In view of the above observations, the effects of these two drugs on the conjugating systems of bilirubin and harmol were investigated.

## MATERIALS AND METHODS

*Animals.* Adults of both sexes were used unless otherwise stated. In the case of the newborn rats, livers from litters born at the same time were pooled in the preparation of the microsomal fractions.

*Enzyme assays.* The activities of bilirubin glucosyl- and glucuronyltransferases and harmol glucuronyltransferase were measured by the procedures of Wong.[3,12,13] In the experiments where animals were pretreated with drugs, adult femals animals were used and microsomal fractions were prepared from individual rats as follows: a 20% (w/v) homogenate, obtained from 3 g of liver was centrifuged at 15,000 *g* for 30 min in the Sorvall Superspeed Automatic Refrigerated Centrifuge (Model RC2-B). The supernatant was re-centrifuged at 90,000 *g* for 90 min in the Spinco Ultracentrifuge (Model L). The microsomal pellet so obtained was redissolved in 2 ml of cold isotonic KCl; this was then dialyzed against 10 mM EDTA, disodium salt, which had been adjusted to pH 8·2. Aliquots of 0·5 ml of the dialysate were stored frozen until used.

*Protein determination.* The protein concentrations in the enzyme preparations were measured by the procedure of Lowry *et al.*[14]

*Drug pretreatment.* (a) Phenobarbitone. Three groups of three rats each were used. The control animals were fed a diet containing 600 g flour, 200 g dried milk, 30 g dried yeast powder and 10 g NaCl, while each of the two experimental groups received the same food mixed with phenobarbitone. The drug was given at a dose of 12 and 65 mg/kg body wt./day, for 6 days. The animals were sacrificed on the seventh day and the hepatic UDP-glucuronyltransferase activity was measured in the homogenate, using harmol as the substrate. The experiment was repeated with three groups of five mice each similarly treated, but the doses of phenobarbitone given were 15 and 75 mg/kg body wt./day. In the third experiment, the rats received the drug (80 mg/kg body wt./day) for 5 days. Both bilirubin glycosyltransferases and harmol glucuronyltransferase were measured, the latter enzyme was assayed in the supernatant after centrifugation of a 6% (w/v) homogenate at 15,000 *g* for 20 min.

(b) "13B" Mixture. Each packet of drug, weighing about 70 g was boiled in 1 l. of water for 3 hr; this is a normal procedure adopted for human consumption. The solution was then decanted and the filtrate made up to 2 l. This solution was given as drinking water, a fresh lot of 100 ml being given daily to a group of three rats. The animals drank this herbal solution without any apparent ill-effects. The enzyme assays were performed after feeding the rats for 22 and 40 days.

*Effects of* "13B" *mixture* in vitro. For this purpose, a four-times more concentrated solution of "13B" mixture than that used in the feeding experiment was used after centrifugation at 90,000 *g* for 90 min and adjusting to pH 8·2. Fractions of 25–100 $\mu$l of this clear but coloured (brown) solution were tested for its effects on the bilirubin transglucuronidating system.

## RESULTS

*Interspecies comparisons.* The specific activities of bilirubin glucuronyl- and glucosyltransferases of various animals are shown in Table 1. For all the animals investigated, the conjugation with glucuronic acid appeared to be higher than that with glucose when the same enzyme preparation was used for both assays.

TABLE 1. COMPARISON OF BILIRUBIN UDP-GLUCURONYL- AND GLUCOSYLTRANSFERASES IN VARIOUS ANIMALS

| Species | Sex | Specific activity, μg bilirubin "equivalents" conjugated per hr per mg microsomal protein: Glucuronyl-transferase (A) | Glucosyl-transferase (B) | A/B |
|---|---|---|---|---|
| Rat | male | 8·2* | 1·7† | 4·8 |
| | female | 11·5* | 2·4† | 4·8 |
| Mouse | male | 7·67 | 0·73 | 10·5 |
| | female | 9·35 | 1·19 | 7·8 |
| Cat | male | 2·04 | 0·91 | 2·2 |
| Hamster | male | 3·36 | 0·54 | 6·2 |
| | female | 3·25 | 0·42 | 7·7 |
| Guinea-pig | male | 0·67 | 0·15 | 4·5 |
| | | 0·40 (60 min assay) | | |
| | female | 0·61 | 0·08 | 7·6 |
| | | 0·41 (60 min assay) | | |
| Man | male | 1·30 | 0·24 | 5·4 |
| | | 0·71 (60 min assay) | | |

Experimental details are given in refs. 3 and 12. Incubation times for glucuronyl- and glucosyltransferases are 30 and 60 min, respectively, unless otherwise indicated.

* Values reproduced from ref. 12.

† Values reproduced from ref. 3.

*Activities of bilirubin glycosyltransferases in young rats.* The results are shown in Table 2. A deficiency of both glycosyltransferases is evident when compared to the corresponding values obtained for the adult rat. There was a progressive development of these enzymes 10 day post-partum until about 50 per cent of the adult value was reached four weeks after birth.

TABLE 2. ACTIVITIES OR BILIRUBIN GLYCOSYLTRANSFERASES IN YOUNG RATS

| Age in days | No. of animals | Specific activity, μg bilirubin "equivalents" conjugated per hr per mg microsomal protein: Glucuronyl-transferase | Glucosyl-transferase |
|---|---|---|---|
| 10 | 10 | 3·77 (45·7%) | — |
| 11 | 9 | 3·70 (44·8%) | 0·9 (39%) |
| 28 (male) | 3 | 4·22 (51·2%) | 1·0 (43·3%) |
| 28 (female) | 3 | 4·63 (56·1%) | 1·03 (44·6%) |

Values in parentheses represent percentages of adult values of glucuronyltransferase, 8·25 ± 0·27 (mean ± S.D.) calculated from 24 determinations and of glucosyltransferase, 2·31 ± 0·1 (mean ± S.D.) calculated from 12 determinations.

*Effects of drugs.* (a) Phenobarbitone. This drug, mixed in the diet, when fed to rat produced an activation of the glucuronyltransferase when harmol was used as the substrate. The stimulation of this conjugating enzyme was shown both in the rats and in the mice (Tables 3 and 4). The increase in enzyme activity appeared to be dose dependent (Table 3). A stimulation of the bilirubin glycosyltransferases was also demonstrated but the degree of stimulation was less than that obtained for the harmol conjugating system (Table 4).

TABLE 3. EFFECTS OF PHENOBARBITONE ON HEPATIC UDP-GLUCURONYL-TRANSFERASE OF RATS AND MICE

| Species | Drug pretreatment | Activity* (nmoles harmol glucuronide conjugated per hr per mg liver) |
|---|---|---|
| Rat | Control | 1·49 (3) |
| | Phenobarbitone (12 mg/kg body wt.) | 2·14 (3) |
| | Phenobarbitone (65 mg/kg body wt.) | 2·65 (3) |
| Mouse | Control | 2·93 ± 0·12 (5) |
| | Phenobarbitone (15 mg/kg body wt.) | 4·51 ± 0·42 (5) |
| | Phenobarbitone (75 mg/kg body wt.) | 7·02 ± 0·17 (5) |

* Experimental details are given in ref. 13. Enzyme activity was measured in freshly prepared homogenate. Results are expressed as mean or mean ± S.E.; numbers of animals are given in parentheses.

TABLE 4. EFFECTS OF PHENOBARBITONE ON HARMOL GLUCURONYLTRANSFERASE AND BILIRUBIN GLYCOSYLTRANSFERASES OF RAT LIVER

| | Enzyme activities* | | |
|---|---|---|---|
| | Harmol glucuronyl-transferase | Bilirubin glucuronyl-transferase | Bilirubin glucosyl-transferase |
| Control (6) | 11·36 ± 0·21 | 8·35 ± 0·30 | 1·88 ± 0·13<br>1·29 ± 0·06† |
| Phenobarbitone (6) | 18·85 ± 1·91 | 9·15 ± 0·26 | 2·36 ± 0·10<br>1·57 ± 0·12† |

* Activities of harmol glucuronyltransferase and bilirubin glycosyltransferases are expressed as nmoles harmol glucuronide conjugated per hr per mg protein (mean ± S.E.) and as μg bilirubin "equivalents" conjugated per hr per mg microsomal protein (mean ± S.E.). Numbers of animals are given in parentheses.

† Assays were repeated as control values were lower than values obtained in other experiments.

*Effects of* "13B" *mixture.* This Chinese drug did not show any action on bilirubin glucuronyltransferase, even after prolonged feeding (Table 5). When added *in vitro*, the herbal solution "appeared" to have an inhibitory effect on the transglucuronidation of bilirubin. However, it was found that the formation of the azopigment of bilirubin glucuronide was also decreased when the drug was added at various dilutions to the control tubes immediately before or after the termination of the reaction with the diazo reagent. The dependence of the assay procedure on the diazotization reaction is therefore a limitation in the investigation of the effects of this drug on the conjugation of bilirubin *in vitro*.

TABLE 5. EFFECTS OF "13B" MIXTURE ON BILIRUBIN GLUCURONYLTRANSFERASE OF RAT LIVER

| | Days of pretreatment | Bilirubin glucuronyl-transferase* |
|---|---|---|
| Control (5) | 22 | 3·74 ± 0·15† |
| "13B" (5) | | 3·65 ± 0·21 |
| Control (5) | 40 | 8·15 ± 0·20 |
| "13B" (5) | | 7·67 ± 0·54 |

* Enzyme activity is expressed as μg bilirubin "equivalents" conjugated per hr per mg microsomal protein (mean ± S.E.). Numbers of animals are given in parentheses.

† In this set of experiment only half of the concentration of UDPGA was used but the time of incubation was increased from 30 to 60 min.

## DISCUSSION

A comparative study of the activities of bilirubin glucuronyl- and glucosyl-transferases showed that in all the species of animals investigated, conjugation with glucuronic acid seemed to be higher than that with glucose. As discussed in a previous paper,[3] this comparison should be interpreted with caution because of the limitations imposed by the assay procedure which is dependent on the diazotization reaction. There was no correlation between the two transferases; ratios of glucuronyl- to glucosyl-transferases ranged from 2·2 in the cat to 10·5 in the mouse. The cat which has been shown to have a defective transglucuronidating capacity[4,5,15] was able to form bilirubin glucoside at a rate comparable to that of the mouse. It is conceivable that this alternative route of conjugation may be a more significant detoxification process in the cat, especially when the hepatic level of UDPG (as measured in various animals[16] and possibly also in the cat) is relatively high compared to that of UDPGA. However, it is premature at this stage to assess the contribution of this conjugation reaction to the overall detoxification mechanism in any animal as this metabolic pathway has not been extensively explored, being hitherto believed to be confined to the invertebrate systems.[17] It may assume a more active role in some animals like the cat, as discussed above, and in the rat where comparative kinetic results[3] seemed to indicate that transglucosylation of bilirubin could be of considerable importance.

Both the glycosyltransferases exhibited essentially similar properties with respect to pH optimum, requirements for $Mg^{2+}$ ions and $K_m$ values for their respective uridine donors.[3,12] They were also demonstrated to be low in the young rat. However, further studies are necessary to ascertain whether the same enzyme is involved in the two reactions, although the different $K_m$ values of these enzymes for bilirubin[3] seemed to suggest otherwise.

Several clinical studies have shown that the administration of phenobarbitone resulted in a decrease in the bilirubin concentration in newborn infants.[18–21] The beneficial action of this drug has been attributed to the induction of glucuronyltransferase.[22] Most of these studies were carried out with unnatural substrates,[23–25] the choice of which has obvious and recognized drawbacks. A recent study using bilirubin as substrate showed no change in the specific activity of bilirubin glucuronyl-

transferase of guinea-pig liver after phenobarbitone treatment.[6] This observation differed from the results reported by Halac and Sicignano[26] and those presented in this paper. A species difference could be responsible for this discrepancy.

Though an enhancement of both the bilirubin conjugating systems by phenobarbitone has been demonstrated in animal experiments, it does not warrant the recommendation of this drug as a therapeutic agent for neonatal hyperbilirubinemia. Firstly, even with bilirubin as substrate, results obtained have not been consistent. Normal levels of this enzyme, measured in different animals differ depending on the assay procedure, e.g. the activities in four animals, as reported by Potrepka and Spratt,[6] in decreasing order are as follows: rat, guinea pig, mouse and cat. This is in contrast to that obtained in this study (i.e. rat, mouse, cat and guinea-pig). Secondly, to what extent would the different assay conditions reflect the functional level of glucuronyltransferase *in vivo* has been questioned.[27] Studies have been performed on both "native" and activated enzymes with invariably different results. It was therefore recommended that parallel studies should be carried out.[28] Thus, with this lack of correlation in enzymic studies which arose essentially from the use of (a) different species of animals (b) different aglycones and (c) different enzyme preparations and assay procedures, animal experimentation could hardly be extrapolated to human physiology. Besides, phenobarbitone has not been uniformly effective in the post-natal treatment of neonatal hyperbilirubinemia.[29–31] The adverse effects of phenobarbitone on other enzyme systems[32] and on normal growth and development[33,34] must be taken into consideration in the studies of action of drugs in the newborn, who are particularly sensitive to the toxic effects of pharmacologic agents.[35,36] Though the "13B" mixture (a drug commonly consumed by Chinese pregnant mothers, and whose chemical composition is probably unknown[9]) had no appreciable effect on the bilirubin glucuronyltransferase of adult female rats, any deleterious action of this drug on the newborn animals remain to be investigated.

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