Biochemical Pharmacology, Vol. 24, pp. 103–107. Pergamon Press, 1975. Printed in Great Britain.

UDP GLUCURONYLTRANSFERASE AND PHENOLSULFOTRANSFERASE FROM RAT LIVER *IN VIVO* AND *IN VITRO*

CHARACTERIZATION OF CONJUGATION AND BILIARY EXCRETION OF HARMOL *IN VIVO* AND IN THE PERFUSED LIVER

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(*Received* 1 *April* 1974; *accepted* 28 *May* 1974)

Abstract—Harmol (7-hydroxy-1-methyl-9H-pyrido-(3,4b)indol) is excreted in bile and urine of rats after an i.v. dose of 20 μmoles/kg in the form of harmol-sulfate and harmol-glucuronide. Ligation of the kidneys caused a compensatory higher biliary excretion of the conjugates. In the isolated perfused rat liver harmol was presented to the liver at two doses: 3 and 15 μmoles. At the lower dose equal amounts of harmol-sulfate and harmol-glucuronide were present in bile. Due to the five-fold increase in harmol dose, a three-fold increase in the biliary excretion of harmol-sulfate was observed but an eleven-fold increase in that of harmol-glucuronide, during the two hours of the perfusion. This effect can be explained by *in vitro* kinetic data on phenolsulfotransferase (EC 2.8.2.1) and UDP glucuronyltransferase (EC 2.4.1.17) and by the situation of competition of these enzymes for the same substrate harmol *in vivo*. The results suggest a model of harmol conjugation and elimination in which: (1) with increasing substrate supply glucuronidation is increased relative to sulfation; (2) harmol-glucuronide, once formed, is rapidly excreted in bile; and (3) harmol-sulfate is excreted in bile at a low rate, such that this step is rate limiting.

Many endogenous compounds and xenobiotics are converted in mammalian liver to both sulfate and glucuronide conjugates; these are subsequently eliminated from the body in bile or urine [1, 2]. Harmol (7-hydroxy-1-methyl-9H-pyrido-(3,4b)indol) has previously been shown to be eliminated in bile and urine in the form of both harmol-sulfate and harmol-glucuronide, after intravenous injection in the rat [3]. Thus, when harmol is given to the rat, *in vivo* two enzymes in rat liver are competing for the same substrate and the ratio of the amount of harmol-glucuronide to harmol-sulfate excreted in bile and urine gives information on the outcome of this competition. Therefore, in the process of elimination of harmol, conjugation to the sulfate form on one hand, and the glucuronide form on the other is, due to this competition, necessarily rate limiting which is reflected in the ratio of glucuronide to sulfate conjugate excreted.

It is possible to measure sulfation and glucuronidation of harmol *in vitro* in a simultaneous incubation at the same dilution of the postnuclear supernatant from rat liver homogenate (Mulder, submitted for publication). In this way the use of harmol as substrate makes it possible to compare properties of both conjugating enzymes observed *in vitro* with those found *in vivo* or in an isolated perfused rat liver. Thus, the physiological relevance of the findings *in vitro* can be tested *in vivo*.

In the present paper we report our findings on the conjugation and subsequent elimination of harmol in the rat *in vivo* and in the isolated perfused rat liver. The well-known advantage of an isolated perfused liver is, of course, that several complicating interactions *in vivo* can be avoided because the compound under investigation is presented to the liver only. Characteristics of harmol conjugation and elimination are reported. The results provide evidence on rate limiting steps in the conjugation and biliary elimination of harmol-sulfate and harmol-glucuronide, and show that properties as determined previously *in vitro* in a postnuclear rat liver supernatant (Mulder, submitted for publication) can be observed in the perfused rat liver.

MATERIALS AND METHODS

Chemicals. Harmol-HCl was obtained from Fluka (Buchs, Switzerland).

Bile cannulation experiments. Male rats (Wistar, 280–350 g) were used in these experiments. They had free access to food and water. The experiments started between 10 and 11 a.m. Bile cannulation and collection of urine from the bladder at the end of the experiment were performed as described before under pentobarbital (Nembutal®) anesthesia [3, 5]. Injections were in the *vena jugularis externa* in a volume of 0·25 ml/100 g body wt.

Determination of harmol conjugates. This was performed as described before [3], modified from published methods [6, 7].

Isolated perfused rat liver. The equipment and method applied in the perfusion experiments were those reported by Meyer and Weitering [4] with one important modification. The perfusion fluid in the present experiments was composed of Krebs–albumin buffer, pH 7·40 to which sheep erythrocytes were added. By centrifugation erythrocytes from 80 ml sheep blood were isolated and washed twice with 0·9% (w/v) NaCl solution under sterile conditions. Between 30 and 35 ml erythrocytes were obtained this way. These were added to 100 ml Krebs–bicarbonate–albumin buffer, pH 7·40 [4], containing 2 mg/ml glucose and 25 μg/ml ampicillin. About 90 ml medium was used in the perfusion and it was recirculated through the liver via the portal vein. The duration of the perfusion was 2 hr. When perfusion flow through the liver had stabilized at 20 ml/min harmol was added to the perfusion medium at either a low dose (3 μmoles or 0·7 mg) or a high dose (15 μmoles or 3·5 mg). Bile was collected in successive 15 min fractions and blood samples were taken at 15 min intervals.

"Plasma" of the perfusion fluid was obtained by centrifugation. The clear supernatant was heated for 1 min in a boiling water-bath and thereafter put on ice. Denatured protein was removed by centrifugation and 20 μl of the supernatant was applied to a thin layer chromatography (t.l.c.) plate and chromatographed as described before [3]. For chromatography of bile, 2 μl at the high dose of harmol and 5 μl at the low dose were applied to the t.l.c. plate. Separation and estimation of harmol-sulfate and harmol-glucuronide were performed as described before [3].

Statistical analysis. Statistical evaluation of differences were performed according to Wilcoxon's method [8].

RESULTS

Biliary excretion of harmol in vivo *in rats with intact or ligated kidneys.* Because we wanted to study the metabolism of harmol in the isolated perfused rat liver in which no urinary elimination of the harmol conjugates is possible, we have studied first the biliary excretion in animals with ligated kidneys. The results are shown in Fig. 1 and Table 1. In rats with intact kidneys 23 per cent of the dose was excreted in 2 hr in urine as harmol-sulfate and about 4 per cent as harmol-glucuronide (Table 1). In rats with ligated kidneys the amount of harmol-sulfate excreted in bile was increased from 26 per cent of the dose (in two hours) to 41 per cent and the amount of harmol-glucuronide was increased from 14 to 17 per cent. Thus, most of the conjugates excreted normally in urine were now excreted in bile, in addition to the amounts normally excreted in bile (Table 1). Figure 1 shows that this increase in biliary excretion of harmol-sulfate was particularly significant in the three latter periods of bile collection. In the first 30 min of bile collection the con-

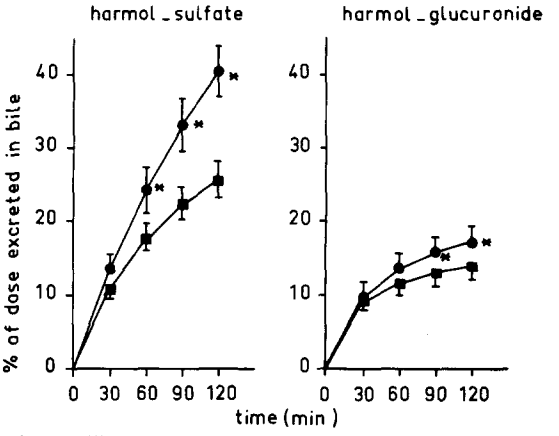

Fig. 1. Biliary excretion of harmol-sulfate and harmol-glucuronide *in vivo* in rats with intact (■) and ligated (●) kidneys. Bile was collected during four 0·5-hr periods. Harmol dose was 20 μmoles/kg i.v. The cumulative percentage of the dose excreted in the form of the harmol conjugates is given. *$P < 0.05$ if compared with biliary excretion in the group with intact kidneys (Wilcoxon).

Table 1. Biliary and urinary excretion of harmol conjugates in rats with intact kidneys and ligated kidneys

	Intact kidneys				Ligated kidneys			
	Harmol-sulfate		Harmol-glucuronide		Harmol-sulfate		Harmol-glucuronide	
	μmoles	%	μmoles	%	μmoles	%	μmoles	%
Excreted in urine	1·42 ± 0·49	23	0·23 ± 0·08	4	0	0	0	0
Excreted in bile	1·61 ± 0·16	26	0·88 ± 0·13	14	2·54 ± 0·22*	41	1·08 ± 0·13	17
Total recovery	4·14 ± 0·49 μmoles (66%)				3·61 ± 0·31 μmoles (58%)			
No. of animals	7				6			

The dose of harmol was 20 μmoles/kg i.v. Bile was collected during four 30-min periods after which urine was collected from the bladder. After the experiments the liver was excised and weighed. The mean liver weight was 3·6 ± 0·1% of body wt (mean ± S.E.M.). Mean body wt of the rats was 313 ± 7 g. Excreted amounts are given in μmoles ± S.E.M. and in percentage of the dose.

* $P < 0.05$ if compared with biliary excretion in the group with intact kidneys (Wilcoxon).

centration of harmol-sulfate in bile was 0·88 ± 0·10 mM; this was 1·15 ± 0·18 mM (means ± S.E.M.) in the rats with ligated kidneys but the difference was not significant. However, during the 2 hr of the experiment a highly significant increase in harmol-sulfate in bile was found as a result of ligation of the kidneys; the increase of harmol-glucuronide in bile was not statistically significant probably due to the low amounts of harmol-glucuronide normally excreted in urine as compared with harmol-sulfate (Table 1).

If bile collection in rats with ligated kidneys was continued for 6 hr after injection a recovery of 105 per cent (94–110) of harmol was found in three rats (the extreme values are given in parentheses). In the first 2 hr 49 per cent (48–51) of the dose was excreted as harmol-sulfate and 19 per cent (16–22) as harmol-glucuronide. During the latter 4 hr 33 per cent (27–35) was excreted additionally as harmol-sulfate and only 3 per cent as harmol-glucuronide.

Perfused rat liver: perfusion medium. Two doses of harmol were added to the perfusion medium: 3 and 15 μmoles, each in three separate experiments. After addition, harmol disappeared rapidly from the perfusion medium due to uptake by the liver. Within 15 min it had disappeared completely. After 15 min both the sulfate and glucuronide conjugate were present in the perfusion medium, at concentrations of about 20 μM (sulfate) and 5 μM (glucuronide) respectively (when 15 μmoles of harmol had been applied to the perfusion). These concentrations gradually decreased during the perfusion to 10 and 2 μM respectively. They were determined in the "plasma"; harmol or its conjugates could not be detected in, or absorbed on, erythrocytes.

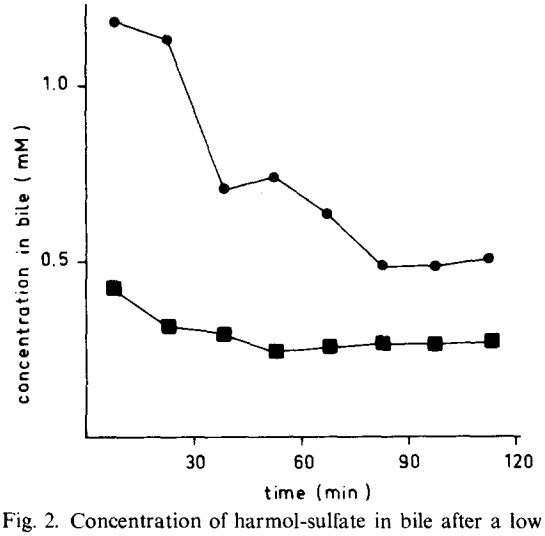

Fig. 2. Concentration of harmol-sulfate in bile after a low dose (3 μmoles; ■) and a high dose (15 μmoles; ●) of harmol. Harmol was added to the perfusion medium of the isolated perfused rat liver at zero time. Bile fractions of 15 min were used for the determinations. Note the difference in ordinate scale between the sulfate and glucuronide results (Fig. 3). The results are the means of three separate experiments.

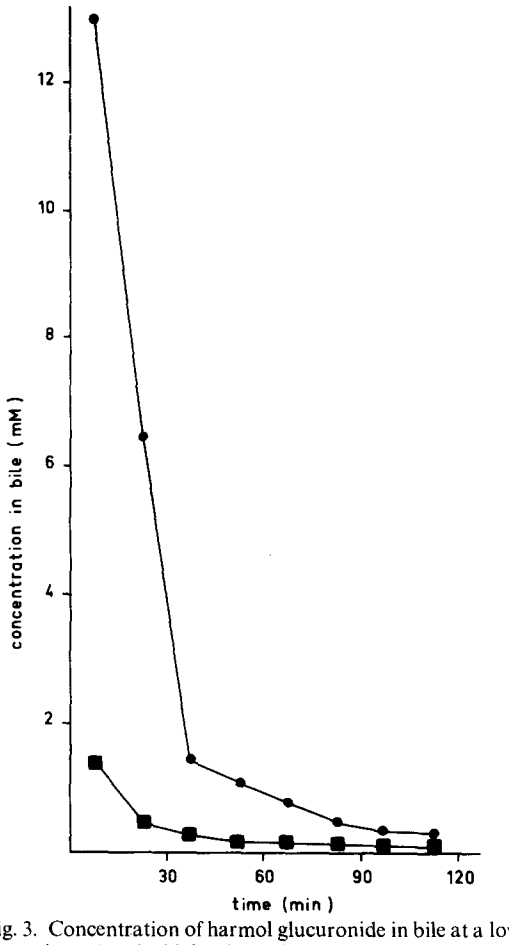

Fig. 3. Concentration of harmol glucuronide in bile at a low (3 μmoles; ■) and a high (15 μmoles; ●) dose of harmol. See also legend to Fig. 2.

Perfused rat liver: biliary excretion of harmol conjugates. Figures 2 and 3 show the characteristics of the biliary excretion of harmol-glucuronide and harmol-sulfate; free, unconjugated harmol was present in bile but only to a negligible degree, in the first period of bile collection. At both doses of harmol, in the first period of 15 min after addition of harmol to the perfusion the concentration of harmol-glucuronide in bile was much higher than that of harmol-sulfate: at 3 μmoles of harmol it was 1·4 (glucuronide), viz. 0·45 mM (sulfate) and at 15 μmoles of harmol it was 13·0, viz. 1·2 mM. The concentration of the glucuronide conjugate fell off rapidly (Fig. 3) after two periods of 15 min, being nearly equal to that of the sulfate conjugate. The concentration of the sulfate conjugate (Fig. 2) decreased much less during the 2 hr of the perfusion: at 3 μmoles of harmol from 0·45 to 0·28 mM and at 15 μmoles of harmol from 1·2 to 0·5 mM. During the second hour of the perfusion the concentration of harmol-sulfate in bile remained rather constant (Fig. 2). It should be noted that at the higher dose of harmol the harmol-sulfate concentration leveled off at 0·50 mM and at the

Table 2. Excretion of harmol-sulfate and harmol-glucuronide in bile during an isolated rat liver perfusion of 2 hr duration

	Dose of harmol			
	3 μmoles		15 μmoles	
	μmoles in bile	% of dose	μmoles in bile	% of dose
Harmol-sulfate	0·43 (0·24–0·55)	14·3	1·26 (1·03–1·62)	8·5
Harmol-glucuronide	0·50 (0·40–0·69)	16·7	5·47 (4·66–5·98)	36·7
Total recovery	0·93 (0·64–1·24)	31·0	6·73 (5·80–7·60)	45·2
Bile produced in 2 hr	1440 mg (1412–1467)		1661 mg (1590–1720)	
Liver wt	13·2 g (12·0–15·6)		13·0 g (12·1–13·6)	

Results are the means of three separate experiments; the range is given in parentheses.

lower dose at 0·27 mM. After 2 hr the concentration of harmol-glucuronide was still decreasing, but was yet slightly higher (0·3 mM) at the higher dose of harmol than at the lower dose (0·1 mM).

When the amounts of harmol-glucuronide and harmol-sulfate expressed in μmoles excreted in bile in the 15 min periods were considered the results are qualitatively the same as those described above for the concentration in bile because bile production was constant during the perfusion. Only in the first two periods of 15 min of bile collection at the higher dose of harmol it was always 15 per cent higher than in the later periods. During the first two periods more glucuronide conjugate than sulfate conjugate was excreted but later on they were excreted at rather similar rates. Again the biliary excretion of the sulfate conjugate stabilized, at a higher level when the higher dose of harmol was used, compared with the lower dose of harmol.

Previously [3] we reported a correlation between the amount of glucuronide in bile and a concomitant extra production of bile (choleresis). In the present experiments we observed that bile production in the liver perfusion with the high dose of harmol (and the higher excretion rate of harmol conjugates) was enhanced compared with the lower dose (and the lower excretion rate) whereas the mean liver weight was the same in both groups (Table 2).

After 2 hr of perfusion the recovery of harmol in the form of its conjugates was very incomplete (Table 2): 31 per cent at the lower dose of harmol and 45 per cent at the higher dose. The data from Table 2 show that the ratio of glucuronide to sulfate conjugate in 2 hr in bile changes considerably with dose of harmol: at 3 μmoles of harmol this ratio is HG/HS = 1·0 whereas at 15 μmoles of harmol this is 4·3. Further, it can be computed that during the perfusion the ratio of glucuronide to sulfate conjugate changes also: at 3 μmoles of harmol from HG/HS = 3·5 (first period) to 0·25 (last period) and at 15 μmoles of harmol from 10·0 to 0·7.

DISCUSSION

The reason we studied harmol conjugation *in vivo* and in the perfused rat liver was to compare properties of UDP glucuronyltransferase and phenolsulfotransferase in these systems (this work) and in the post-nuclear rat liver supernatant (Mulder, submitted for publication). For reasons given in the Introduction the results might also give information on rate limiting steps in the biliary elimination of harmol. Conjugation *in vivo* and in the perfusion can only be studied indirectly by measurement of the amounts of the glucuronide and sulfate conjugates eliminated in bile and urine. This would not be a serious complication if the elimination is not a rate limiting step and if only the sulfation and glucuronidation processes *per se* are rate limiting. If elimination becomes rate limiting the conjugation rate cannot be measured properly because the properties of the elimination process are then determined.

As far as harmol-glucuronide is concerned, in the present experiments there seems to be no indication that its elimination is rate limiting. Only small amounts appear in urine whereas the concentration in bile can become as high as 13 mM, which was only reached at a high harmol load (Fig. 3). In all other experiments much lower concentrations were found in bile. Thus, for harmol-glucuronide, its formation rate seems rate limiting: once it is formed it is readily excreted in bile and, only to a limited extent, it leaks back into the blood and is excreted in urine. In agreement with this we found during the perfusion a lower concentration of harmol-glucuronide in the perfusion medium than of harmol-sulfate.

In the case of harmol-sulfate there are indications that elimination in bile may be rate limiting. *In vivo* a considerable amount of harmol-sulfate is eliminated in the urine. When the kidneys are ligated, twice the normal amount of harmol-sulfate (Table 1) is supplied at the sites for transport from liver cell into bile, presumably "carriers". In the first 30-min period nevertheless the concentration in bile increased only from 0·9 to 1·2 mM (Fig. 1) and the percentage excreted in bile in this period increased from 11 to 13 per cent of the dose. This suggests saturation of the transport process, reflected by a maximum concentration of about 1·2 mM of harmol-sulfate in bile. Possibly the same is seen in the liver perfusion at the high dose of harmol in which during the first two periods of 15 min the concentration in bile remained 1·2 mM (Fig. 2).

At both doses of harmol presented to the perfused

liver we observed a stabilization of harmol-sulfate excretion in bile in the second hour of the perfusion. This indicated that: either the liver contains a high amount of harmol-sulfate which is removed at a constant rate in bile as long as the concentration in the liver does not decrease appreciably (thus, biliary excretion is rate limiting); or harmol-sulfate (and to some extent also harmol-glucuronide) is formed during the second hour of the perfusion at a constant rate from unconjugated harmol which is slowly released from some storage in the liver, beyond reach of both conjugating enzymes. For technical reasons we were not able to determine the amount of harmol and its conjugates in the liver, so we can not solve this problem unequivocally. However, although in the perfusion experiments harmol-sulfate excretion stabilized during the second hour, harmol-glucuronide excretion still decreased during that time (Figs. 2 and 3). If harmol-sulfate were synthesized at a constant rate during that time, the same should apply to the synthesis of harmol-glucuronide and, therefore, also biliary excretion of harmol-glucuronide should be constant; this is not the case. For that reason we prefer the first alternative that rather high amounts of harmol-sulfate may be present in the liver which are eliminated only at a rather slow rate. The alternative explanation, that the concentration of harmol is so low that it is only sulfated, and not glucuronidated to any extent, can, however, not be excluded.

From the ratios of harmol-sulfate to harmol-glucuronide produced we can conclude that at a high concentration of harmol (the dose of 15 μmoles *versus* the dose of 3 μmoles) its glucuronidation increases relative to its sulfation; at a relatively low dose of harmol, sulfation may be already saturated, whereas glucuronidation can still increase. This would be in agreement with the findings reported previously on kinetic characterization of harmol conjugation in a rat liver post-nuclear supernatant [3, and Mulder, submitted for publication]. The K_m of phenolsulfotransferase for harmol was found to be less than 15 μM whereas UDP glucuronyltransferase had an apparent K_m of 150 μM for harmol. The results with the perfused liver thus confirm the physiological relevance of these *in vitro* findings. A similar finding was reported by Minck *et al.* [9] who measured glucuronidation and sulfation of *p*-nitrophenol in the perfused rat liver. An alternative explanation, namely that harmol *in vivo* might activate its own glucuronidation at high concentrations (as has been shown to occur for the glucuronidation *in vitro* of *o*-aminophenol and *p*-nitrophenol in not-activated beef liver microsomes), can not be excluded by the present findings, although we have no data supporting it.

The recovery in bile of harmol in the form of its conjugates after two hours of perfusion is very low. The fact that it is higher (45 per cent recovery) at the higher dose of harmol than at the lower dose (31 per cent recovery) is due to the disproportionately greater increase in glucuronidation of harmol (Table 1). That at a five-fold increase of harmol dose the amount of harmol-glucuronide can increase eleven-fold is due to the competition of phenolsulfotransferase and UDP glucuronyltransferase for the same substrate. At the lower dose of harmol UDP glucuronyltransferase was at a disadvantage. The higher recovery might thus be due to the quicker biliary excretion of the glucuronide conjugate.

From comparison of Tables 1 and 2 it appears that *in vivo* harmol is sulfated to a much higher degree than in the perfused liver. This might indicate impaired liver function in the perfusion. It might, however, also mean that harmol enters the liver *in vivo* at a slower rate (due to e.g. redistribution *in vivo*) than the perfused liver; thus the harmol concentration in liver *in vivo* would be lower and sulfation is favoured relative to glucuronidation.

In conclusion, the model we propose for harmol conjugation and biliary excretion by rat liver has the following features: (1) With increasing substrate supply glucuronidation is increased relative to sulfation. (2) Harmol-glucuronide, once formed, is rapidly excreted in bile. (3) Harmol-sulfate is excreted in bile at a low rate, such that this step is rate limiting and may lead to accumulation of harmol-sulfate in the liver; if urinary elimination is lost it will be slowly excreted from liver into bile.

Acknowledgement—We are indebted to Mr. M. Houwertjes for technical assistance.

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