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KINETICS OF SULFATION AND GLUCURONIDATION OF HARMOL IN THE PERFUSED RAT LIVER PREPARATION

DISAPPEARANCE OF ABERRANCES IN GLUCURONIDATION KINETICS BY INHIBITION OF SULFATION

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Abstract—Harmol is conjugated by glucuronidation and sulfation when it is given to the rat *in vivo*. In the once-through perfused rat liver preparation glucuronidation of harmol shows kinetic aberrances [Pang *et al.*, *J. Pharmac. exp. Ther.* **219**, 134 (1981)]. In order to further delineate the mechanism behind this, sulfation was inhibited to about 10% of control by 2,6-dichloro-4-nitrophenol. The loss of sulfation was compensated by an increase in the rate of glucuronidation, keeping the total clearance by the liver virtually constant in spite of the loss of sulfation. The inhibition of sulfation eliminated the previously observed lag-phase in the kinetics of glucuronidation; the rate of glucuronidation was now almost linear with the input concentration of the substrate harmol. The constant clearance of harmol in spite of inhibition of sulfation, the occurrence of the lag-phase in glucuronidation in the presence of sulfation, and the disappearance of this lag-phase in the absence of sulfation can be explained by either diffusion-limited metabolism of harmol or a heterogeneous sub-lobular distribution of the sulfating and glucuronidating systems. Activation of glucuronidation by harmol at high concentration can be excluded.

Phenolic compounds are usually metabolized in the liver of vertebrates by sulfation and glucuronidation [1, 2]. The kinetics of these conjugation reactions at steady-state have been studied *in vivo* [3], in the once-through perfused rat liver [4–6] and in isolated hepatocytes [7–10]. Sulfation and glucuronidation are competing reactions for a common substrate: compensatory increases in glucuronidation rates occur with inhibition or saturation of sulfation [11, 12]. Usually the affinity of the sulfotransferases for their substrates is higher than that of UDP-glucuronosyltransferase, but the reverse is true for their enzymatic capacity, V_{max} [13]. Therefore, at low substrate concentration, sulfation will predominate and at high substrate concentration glucuronidation is more important [2, 14, 25].

The kinetics on the rates of sulfation and glucuronidation at steady-state in both the perfused rat liver and the rat *in vivo* revealed that the rate of glucuronidation increased more than proportionally with increasing substrate concentration or dose [3–9]. Several explanations have been provided for this apparent acceleration of glucuronidation: (a) a different intracellular localization of the enzyme systems for sulfation (cytosolic) and glucuronidation (endoplasmic reticulum) [4]; (b) a different localization in the liver lobulus for sulfation (periportal hepatocytes, zone 1) and glucuronidation (centrilobular hepatocytes, zone 3) [6]; (c) an activation of UDP-glucuronosyltransferase by increasing substrate concentrations [9, 16]; and (d) the existence of a diffusional barrier for the uptake of the substrate by the liver resulting in a non-linear relationship between the extra- and intracellular substrate concentration under conditions when sulfation becomes saturated [17].

The unusual behaviour of glucuronidation in the presence of its competing reaction, sulfation, can be better understood when sulfation is eliminated from the system. This study is an investigation on the steady-state kinetics of glucuronidation of the phenolic compound harmol, under conditions where sulfation was reduced to about 10% of its control value; this low level of sulfation was attained by use of a specific inhibitor of sulfation, 2,6-dichloro-4-nitrophenol (DCNP) [18]. The long term perfusion of DCNP to the perfused rat liver was without deleterious effects [11]. The present results indicate that the aberrancy in the kinetics of glucuronidation observed in the presence of sulfation, disappeared in the absence of sulfation. This indicates that the unusual behaviour of glucuronidation cannot be caused by substrate activation of UDP-glucuronosyltransferase.

MATERIALS AND METHODS

Materials. Harmol hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO); 2,6-dichloro-4-nitrophenol was obtained from Aldrich Europe (Beerse, Belgium); dried, demineralized

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bovine serum albumin was purchased from Poviet Products (Oss, The Netherlands); silica gel thin-layer chromatography plates (60 F-254) were from Merck (Darmstadt, F.R.G.); Dextran T-40 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). General tritium-labeled harmol was prepared by tritium gas exposure labeling by New England Nuclear (Boston, MA) and was further purified by high performance liquid chromatography as described elsewhere [11].

Composition of the perfusion medium. This medium [6] consisted of 15% (v/v) washed sheep erythrocytes, 0.3% glucose (w/v), 1% bovine serum albumin (w/v), 3% Dextran T-40 (w/v) and a constant concentration of [^{3}H]harmol in Krebs–Ringer bicarbonate solution (pH 7.4). 2,6-Dichloro-4-nitrophenol was dissolved initially in saline to a concentration of 10 mM by addition of 4 N NaOH. The pH of this solution was adjusted to pH 7.4 with 1 N HCl and aliquots of this solution were added to the perfusion medium to furnish the desired concentration of DCNP (0, 20, 40 and 60 μM) in the medium.

The liver perfusion system. The perfusion apparatus was identical to that described previously [6]. Male Wistar rats (290–310 g) that were fed *ad libitum* were used. Sodium pentobarbital (60 mg/kg i.p.) was used for anesthesia prior to surgery. Surgical techniques were identical to those previously described [19]. The livers were perfused *in situ* in a non-recirculating fashion; medium was pumped from a reservoir at a flow rate of 10 ml/min through a 6 m long Silastic® tubing (Dow-Corning Corp., Midland, MI) inside a perspex cylinder which was constantly gassed with carbogen (95% O_2, 5% CO_2) and oxygen for oxygenation. The pH of the input medium was constantly monitored and adjusted to pH 7.4 by adjusting the ratio of carbogen to oxygen. The hydrostatic pressure of the perfusion system was also determined by an open vertical tubing that was connected between the pump and the liver.

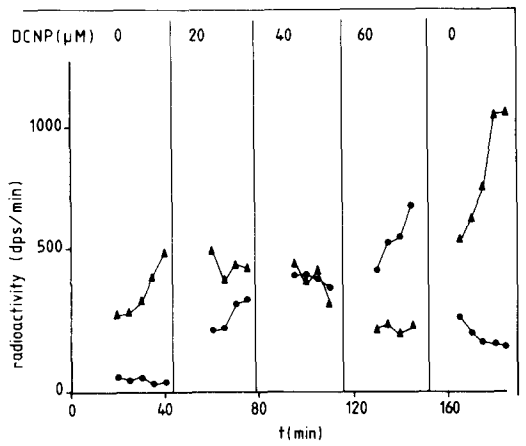

Fig. 1. Effects of various concentrations of 2,6-dichloro-4-nitrophenol (DCNP) on the conjugation of harmol. A constant tracer concentration of [^{3}H]harmol was delivered once-through (10 ml/min/liver) to the perfused rat liver. The concentration of DCNP in the perfusion medium was increased step-wise as indicated. Harmol sulfate in the effluent medium (▲), harmol glucuronide in the effluent medium (●). Excretion of conjugate in bile:

Time (min):	0–40	40–75	75–110	110–150	150–190
Harmol sulfate (% of dose):	29	20	11	6	13
Harmol glucuronide (% of dose):	2	17	45	44	22

Analyses of harmol and its conjugates. Quantitation of unconjugated harmol was performed by an extraction method into ethylacetate as previously described [6]. Separation of harmol conjugates in bile and perfusate plasma was achieved by thin-layer chromatography [6, 20].

Protein binding of harmol. Protein binding of harmol (10–200 μM) was determined in the presence of DCNP (60 μM) in perfusate by equilibrium dialysis [6].

RESULTS

Protein binding of harmol

The effect of DCNP on protein binding of harmol was assessed. DCNP (60 μM) did not affect the degree of binding of harmol (10–200 μM) to bovine serum albumin (1% in medium); the binding was 40% over the whole harmol concentration range tested.

The effective concentration of DCNP as an inhibitor of sulfation

In as much as sulfation proceeds maximally at tracer harmol concentrations, the rat liver preparation was perfused with a tracer concentration of [^{3}H]harmol. The concentration of DCNP, however, was increased stepwise (0, 20, 40, 60 and 0 μM) at 40 min intervals. The rate of efflux of harmol sulfate in the effluent perfusate decreased at increasing DCNP concentrations in the perfusion medium (Fig. 1). An increased efflux of harmol glucuronide in the perfusate, however, compensated for this inhibition of sulfation. Similar changes were seen in bile (Fig. 1). Despite that a steady-state was not approached in this experiment, 60 μM DCNP reduced the rate of sulfation considerably. In further experiments it was found that the reduction of the rate of sulfation was about 90% when compared to perfusion without DCNP (see below and Ref. 6). During the last period of perfusion (control) where DCNP was omitted, sulfation increased again and this was accompanied by a reduction in the glucuronidation rate. This observation was consistent with our previous findings on the reversibility of DCNP as an inhibitor of sulfation (11). For the further studies we have used 60 μM DCNP to inhibit harmol sulfation.

Cholestasis due to harmol glucuronide

Because inhibition of sulfation was compensated by an increase in glucuronidation, harmol glucuronide production was accelerated in our experiments where DCNP (60 μM) was included in the perfusion medium. The cholestatic effect of harmol glucuron-

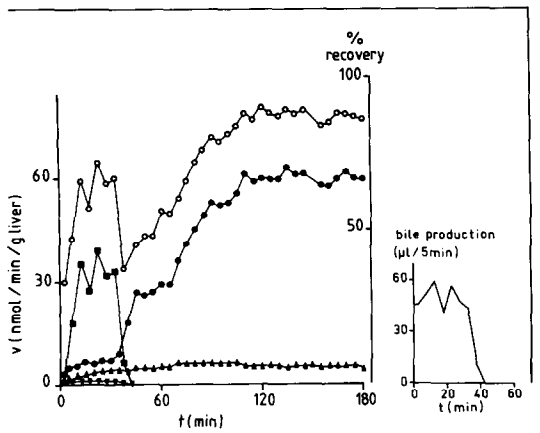

Fig. 2. Cholestasis during simultaneous perfusion (10 ml/min/liver) with 100 μM harmol and 60 μM 2,6-dichloro-4-nitrophenol into the rat liver preparation. Harmol glucuronide in bile (■), harmol glucuronide in effluent medium (●), harmol sulfate in bile (▼), harmol sulfate in effluent medium (▲), recovery (○) (recovery is the sum of unchanged harmol and its conjugates recovered from bile and effluent medium as a fraction of the rate of input of harmol).

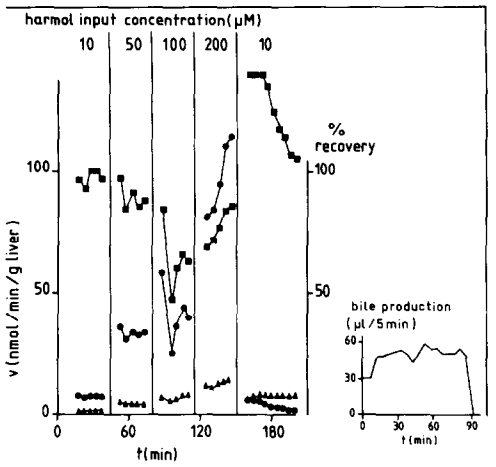

Fig. 3. Sulfation and glucuronidation of harmol at various input harmol concentrations in the presence of 60 μM 2,6-dichloro-4-nitrophenol (DCNP) in the single-pass perfused rat liver. Harmol was delivered at the concentration indicated to the once-through perfused rat liver preparation at a rate of 10 ml/min/liver. DCNP was omitted during the last perfusion period (only 10 μM harmol). The combined rates of excretion in bile and efflux in the perfusion medium are given. Harmol glucuronide (●), harmol sulfate (▲), recovery (■). (Recovery is taken as the sum of unchanged harmol and its conjugates recovered from bile and effluent medium as a fraction of the rate of input of harmol.)

ide [21] surfaced at input harmol concentration of 100 μM, in contrast to our previous observation of the phenomenon at input harmol concentration of 200 μM in the absence of DCNP [6]. In these experiments where harmol (100 μM) and DCNP (60 μM) were delivered simultaneously into the once-through perfused rat liver preparation, both harmol glucuronide and harmol sulfate were detected in perfusate and bile for the first 40 min after the commencement of the experiment (Fig. 2); harmol glucuronide was the predominant species in bile, while harmol sulfate was present mainly in the effluent perfusate. At about 40 min after perfusion, however, total cessation of bile flow occurred; harmol glucuronide gradually increased in the effluent perfusate, and reached a steady-state within the next 70 min. Also, during cholestasis, harmol sulfate increased gradually in the effluent perfusate but the rate of sulfation (sum of excretion rate in bile and rate of efflux in perfusate in steady-state) remained unaltered. Because of the decreased excretion of harmol glucuronide immediately after the stoppage of bile flow, the total rate of excretion of conjugates, as a fraction of drug loss (drop in harmol concentration across the liver multiplied by hepatic blood flow rate) decreased transiently. Therefore, no steady-state was present during this period of decreased output of conjugates. Despite this decrease in the rate of excretion (as per cent of drug loss), the hepatic extraction ratio of harmol remained unchanged throughout the entire experiment (Fig. 2), indicating that the rate of metabolism was not affected by the cholestasis.

Glucuronidation of harmol in the presence of DCNP, an inhibitor of sulfation

Subsequent studies were performed at a constant concentration of DCNP (60 μM), and at a varying input harmol concentration (10, 50, 100 and 200 μM) at 35–75 min intervals. The last period of perfusion (45–60 min) was performed at 10 μM harmol in the absence of DCNP to assess the capacity of the liver to sulfate. In one experiment (study no. 5) DCNP was not omitted in the final period in order to evaluate any deleterious effects on the conjugation systems that might have developed during this very long experiment: the conjugation rates of the initial period were restored.

Sampling from the reservoir during a perfusion period was performed at the beginning and end of the period, and the mean of the determinations was taken as the steady-state input harmol concentration. Sampling of the effluent perfusate during each period (35–75 min) was performed during the last 20 min of perfusion of the period, and five consecutive samples were taken at 5 min intervals. During these 5 min time intervals bile was collected in 5 min fractions.

The effect of DCNP on harmol sulfation was easily apparent. The suppression of sulfation was almost complete (90%) and the stepwise increase in the concentration of harmol (10, 50, 100 and 200 μM) failed to produce significant proportions of harmol sulfate (5–15% of the rate of presentation of harmol). Contrastingly, the inhibition of sulfation was compensated by the production of harmol glucuronide which increased steadily with increasing harmol concentrations (Fig. 3). Cholestasis occurred during the perfusion period of 100 μM of harmol, but this perfusion period was not extended long enough to allow time for a new steady-state because the experiment needed to be kept short (maximum of 3½ hr) for preservation of liver viability as learned from previous experience. Consequently the rate of glucuronidation might be underestimated during this per-

Table 1. Effect of DCNP (60 μM) on harmol conjugation at varying harmol presentation to the once-through perfused rat liver preparation

Study no.	Rate of input of harmol (nmole/min)	Period of perfusion (min)	Steady-state extraction ratio	Rate of formation Harmol sulfate (nmole/min)	Harmol glucuronide (nmole/min)	% Recovery
(1)	100	50	0.82	6	68	92
	500	35	0.80	24	315	88
	1000	40	0.79	36	373*, 606†	62
	2000	35	0.76	114	915	76
	100‡	57	0.84	42	42	116
(2)	100	55	0.87	9	56	78
	500	30	0.88	41	343	90
	1000	40	0.89	97	443*, 620†	65
	2000	40	0.81	152	1054	80
	100‡	60	0.92	13	16	97
(3)	100	55	0.88	13	55	80
	500	35	0.65	32	197	81
	1000	40	0.66	67	313*, 423†	72
	2000	40	0.55	140	711	88
	100‡	60	0.87	71	17	91
(4)	100	40	0.90	16	74	98
	500	35	0.87	42	333	88
	1000	35	0.84	72	397*, 660†	63
	2000	35	0.78	137	1071	82
	100‡	55	0.89	20	20	109
(5)	100	40	0.83	3	47	68
	500	40	0.84	26	242	70
	1000	75	0.79	58	592	86
	2000	40	0.75	106	901	75
	100	45	0.75	7	59	91
(6)	1000	100	0.83	71	615	86
(7)	1000	180	0.79	49	601	86

Rat livers (10 g) were perfused with step-wise increasing concentrations of harmol; at each concentration a steady-state was obtained unless otherwise indicated. The excretion rate of harmol in bile was negligible. The values given are the mean of the three determinations during the final 10 min of each perfusion period with a particular input harmol concentration. The values of study no. 6 and 7 are the mean of the final 40 and 80 min respectively (representing 9 and 17 determinations respectively).

* Steady-state was not achieved for the perfusion period.

† Corrected values, see Discussion.

‡ DCNP (60 μM) was absent in the perfusate.

fusion period because of a smaller total rate of excretion of harmol glucuronide. The decrease in the excretion of harmol glucuronide probably accounted for the smaller total rate of excretion of conjugates as a fraction of the rate of drug loss (% recovery, Table 1). But in one study (study no. 5) where the perfusion period of 100 μM harmol was extended to 75 min, the glucuronide excretion and the total excretion rate of conjugates were higher than in other experiments and recovery was comparable to that of perfusion periods with other input concentrations. However, such long experiments could not be done routinely; study no. 5 was the only successful experiment out of a series of three.

During the final period of perfusion where DCNP was omitted in the medium, the rate of glucuronidation fell below the values obtained during the first period of perfusion while the rate of sulfation increased above the values obtained during the first period of perfusion, indicating that the effects of DCNP on harmol sulfation were transient; harmol conjugation returned rapidly to higher sulfation and lower glucuronidation. Surprisingly, the steady-state hepatic extraction ratio of harmol remained relatively constant during the simultaneous delivery of DCNP and harmol compared with the perfusions with harmol only (final period and Ref. 6). Only a slight decrease in the extraction ratio was observed at 200 μM input harmol concentration. The results of the experiments are summarized in Table 1 and a representative experiment is depicted in Fig. 3.

DISCUSSION

The present study shows that the total steady-state clearance of harmol by the perfused rat liver preparation was not decreased when sulfation of harmol was inhibited by DCNP. The clearance was kept constant by a drastic increase in glucuronidation of harmol that completely compensated for the reduction of sulfation.

The manner in which the glucuronidation rate varied with increases in input harmol concentration was investigated. In studies where DCNP (60 μM) was present in the system when input harmol concentrations were varied from 10 to 200 μM, the glucuronidation rate, when measured at steady-state can be directly related to a given harmol concentration. During the period of perfusion with 100 μM harmol, however, cholestasis occurred; this resulted

in a transient decrease in the excretion rate of harmol glucuronide that was detected by the rates at which this conjugate appeared in bile and perfusate. This transient reduction in rate of efflux of harmol glucuronide may be due to a period of adjustment (Fig. 2). Harmol glucuronide in the hepatocytes may need to be accumulated to a higher level intracellularly before the rate of efflux of this conjugate in the effluent medium reaches the rate of its formation. Then a new steady-state for the glucuronide will be established when the rate of efflux again equals the rate of formation. Because this new steady-state required a long time to be established (almost 100 min, Fig. 2), and because of preservation of the liver viability in the dose studies (studies 1–4), a new steady-state was not approached during the perfusion with 100 μM (perfusion period 35–40 min). Only when this perfusion period was extended to 75 min (but this usually extended the total duration of the experiment too much to preserve liver viability) steady-state was obtained at this input harmol concentration. A correction was therefore made for the rate of glucuronidation for this period (100 μM harmol and 60 μM DCNP in experiments 1–4) by assuming the % recovery (% recovery as a per cent of drug loss) to be the same as the average of the % recovery for the other perfusion experiments, and by taking the difference between the total conjugation rate and the rate of sulfation as the rate of glucuronidation. The values obtained after correcting for the underestimation of harmol glucuronide formation in the 100 μM harmol input concentration period were comparable to those obtained in long term studies

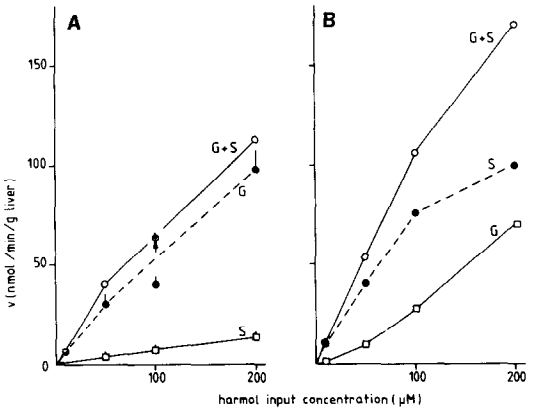

Fig. 4. Rates of glucuronidation (G) and sulfation (S) of harmol at various input harmol concentration in the once-through perfused rat liver preparation; effect of the co-administration of 2,6-dichloro-4-nitrophenol (DCNP). The rat liver was perfused at a flow rate of 10 ml/min/liver with increasing harmol concentrations (10, 50, 100 and 200 μM) in the presence (A) or absence (B) of 60 μM DCNP in the perfusion medium. (A) The rates of conjugation represent the mean of all studies except for the glucuronidation rate at 100 μM harmol: (●) mean of studies no. 1, 2 and 4; (⊙) mean of corrected values (see Discussion) of studies 1, 2 and 4; (▲) mean of studies 5, 6 and 7. The bars on the data points represent the standard deviation. (B) This figure was taken from Ref. 6 where the same harmol input concentrations were delivered to the perfused rat liver preparation in the absence of DCNP.

(study no. 5: 75 min; study no. 6: 100 min; study no. 7: 180 min), (Table 1).

When the rate of glucuronide formation was plotted against the input harmol concentration (Fig. 4), a relatively linear line was obtained, although a slight reduction occurred at high harmol input concentration (200 μM), possibly due to saturation of the glucuronidation system. Moreover, a 'lag-phase' that was observed in our previous studies where harmol (10–200 μM) was presented to the liver in the absence of DCNP [6] was absent in our present study.

The difference in total rate of conjugation (G + S) between Fig. 4A and Fig. 4B is completely due to a lower recovery (15–20% lower) in the experiments of Fig. 4A; the extraction ratio's in the present experiments (Table 1) are very close to those in the previously published experiments of Fig. 4B [6]. Only at the highest harmol input concentration (200 μM) the extraction ratio decreased in the present experiments, presumably due to saturation of glucuronidation.

Several explanations were suggested for the aberrant kinetics of glucuronidation of harmol when sulfation remained intact and served as a competing reaction [6]. The suggestion that substrate activation of UDP-glucuronosyltransferase at higher concentrations of the substrate [9, 16] may be the cause, appears unlikely from the present findings: the 'lag-phase' disappeared in the present study although the same substrate concentrations were used as before [6]. One possible explanation is the existence of a diffusion barrier for the uptake and release of unchanged substrate [17]: saturation of sulfation will cause a non-linear increase in the intracellular substrate concentration as the extracellular harmol concentration is increasing, which increases the rate of glucuronidation because the substrate can only slowly escape the cell due to the diffusion barrier. The same may then be true when sulfation is inhibited: the increase in the intracellular substrate concentration increases the rate of glucuronidation keeping the total rate of metabolism virtually constant. This explanation requires that conjugation is much faster than release of unchanged substrate from the cell. Another plausible explanation is an uneven distribution of the sulfation and glucuronidation activities in different regions of the liver: the centre of enzymic activity of sulfation may be in the periportal region while the centre of enzymic activity of glucuronidation may be posterior to that for sulfation. This can also explain the data, and recent findings on the sulfation and glucuronidation patterns of harmol (50 μM) by normal and retrograde perfusion indeed suggest the possibility [22].

Our present studies have excluded substrate activation of UDP-glucuronosyl-transferase by harmol as possible explanation for the 'lag-phase' in the rate of glucuronidation at increasing harmol concentrations when sulfation remained intact in the system. The role of other mechanisms as causes for the 'lag-phase' is currently investigated.

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