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DOSE-DEPENDENT SHIFTS IN THE SULFATION AND GLUCURONIDATION OF PHENOLIC COMPOUNDS IN THE RAT *IN VIVO* AND IN ISOLATED HEPATOCYTES

THE ROLE OF SATURATION OF PHENOLSULFOTRANSFERASE

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Abstract—The role of enzyme-kinetic parameters of sulfotransferase and UDP-glucuronyltransferase in the balance between sulfation and glucuronidation of various phenolic substrates was studied in the rat *in vivo* after i.v. adminstration and in isolated hepatocytes. A pronounced shift from sulfation to glucuronidation was observed *in vivo* upon increasing the dose of two phenols, harmol and phenol. Similar shifts were found when these compounds were incubated with isolated hepatocytes. However, the shift from sulfation to glucuronidation was small when 4-chlorophenol, or absent when 4-*t*-butylphenol were given *in vivo*. Such shifts were also absent when 4-chlorophenol and 4-*t*-butylphenol were incubated at increasing concentrations with isolated hepatocytes. The *in vivo* results with the various phenols were very similar to the conjugation patterns found in isolated hepatocytes. This suggests that these conjugations in hepatocytes are regulated by similar factors as in the intact animal.

In isolated hepatocytes at most 16 per cent of the available pool of inorganic sulfate was consumed during the incubation. Since Cheng and Levy have shown [*J. biol. Chem.* **255**, 2637 (1980)] that uptake of inorganic sulfate by hepatocytes is very rapid, the present results suggest that the limitation of sulfation of harmol and phenol at increasing dose was caused by saturation of the overall sulfation process by the acceptor substrate, rather than by depletion of inorganic sulfate.

Phenol is both sulfated and glucuronidated in the rat and other mammalian species [1, 2]. At low dose the sulfate conjugate is the major metabolite, while at high doses glucuronidation is quantitatively the more important metabolic pathway for this substrate [3, 4]. A similar dose-dependence of the ratio between glucuronidation and sulfation has been observed with other phenolic substrates *in vivo* or in the perfused rat liver [5–8], and in isolated rat hepatocytes [9–12].

The mechanism of this decrease of sulfation relative to glucuronidation at increasing dose *in vivo* may be a depletion of sulfate at high doses of phenolic substrate [13]: the liver contains a relatively small pool of the sulfate-donating cosubstrate adenosine 3′-phosphate 5′-sulfato-phosphate (PAPS) [14, 15] whereas much more UDP-glucuronic acid is present. However, inorganic sulfate in the blood is immediately available for PAPS synthesis and subsequent sulfation in the liver [15], and only a mild depletion of plasma sulfate has been observed after a high dose of phenol [4, 16]. Moreover, a thorough evaluation of the available data [17] suggests that in many experiments depletion of inorganic sulfate by a substrate for sulfation cannot be the cause of the observed dose-dependent shift in the conjugation pattern because the doses employed were too low. Therefore, other factors have to be considered.

An alternative explanation for the dose-dependent shift in conjugation is based on the observation that *in vivo* K_m values of many phenols are smaller for phenolsulfotransferase than for UDP-glucuronyltransferase [4, 16, 18, 19]. Experiments in isolated hepatocytes with several phenolic substrates at, however, only two substrate concentrations seemed to confirm this idea [9]. A more complete characterization seemed indicated. Since we had found previously that harmol (7-hydroxy-1-methyl-9H-pyrido-[3,4-b]indole), phenol and 4-chlorophenol had a lower K_m for sulfation than for glucuronidation, while the K_m of 4-*t*-butylphenol for sulfation was higher than that for glucuronidation [16, 20], we have used these four compounds in the present study at several dose levels to investigate the relation between the *in vitro* determined K_m-values and in the *in vivo* conjugation patterns.

Another factor that might be involved in the dose-dependent shift in conjugation may be extrahepatic conjugation. So far, the liver is usually considered to be the main site of sulfation and glucuronidation because of its high activities of sulfotransferase and UDP-glucuronyltransferase. Recently, however, it has been shown by Cassidy and Houston [21, 22] that a high first pass conjugation occurred in the lungs when phenol was administered intraveneously, indicating that extrahepatic conjugation contributes to a considerable extent to the conjugation pattern of phenol. Such an extrahepatic conjugation might cause a dose-dependent shift in conjugation if the extrahepatic first pass conjugation could become saturated at increasing dose and another organ with a different ratio between sulfation and glucuronidation would

take over. Therefore, it was of interest to know whether an isolated liver preparation could exhibit the dose-dependent shift observed *in vivo*. This was studied by incubating isolated hepatocytes at various substrate concentrations of the above mentioned phenols.

The present results show that conjugation of three phenols by the liver alone would give rise to the observed dose-dependent conjugation patterns *in vivo*; at moderate doses of the substrate limitation of sulfation is caused primarily by saturation of the sulfotransferase.

MATERIALS AND METHODS

Materials. ^{14}C-labeled phenol was obtained from New England Nuclear, Dreieich, (West Germany) and ^{14}C-labeled 4-chlorophenol and 4-*t*-butylphenol from the Radiochemical Centre, Amersham, (U.K.). [^{3}H]Harmol was produced by New England Nuclear by catalytic tritium-exchange labeling and purified to radiochemical purity by preparative high performance liquid chromatography. Unlabeled phenol, 4-chlorophenol and silicagel-60 F254 thin layer chromatography plates were obtained from Merck, Darmstadt (West Germany); 4-*t*-butylphenol from Aldrich Europe (Beerse, Belgium); harmol and collagenase (Type I) from Sigma (St. Louis, MO, U.S.A.). Picofluor TM 30 Scintillation Medium was from Packard Instrument Company (Downers Grove, IL).

Rats. Male Wistar rats of 200 g body weight were used for *in vivo* experiments with ^{14}C-labeled phenols, and male rats of 300 g body weight for *in vivo* experiments with harmol and for the isolation of hepatocytes. The animals had free access to food and water. Just before the experiments they were anesthesized with sodium pentobarbital (60 mg/kg i.p.).

For the *in vivo* experiments body temperature of the rats was kept between 37.5 and 38.5° by a heating pad. Artificial respiration was applied through a Y-tube in the trachea. To stimulate diuresis during the experiments an infusion of D-mannitol was given through a catheter in the jugular vein. A solution of 75 mg D-mannitol/ml in aqueous 0.9 per cent (w/v) NaCl was infused at a rate of 1.9 ml/hr. When harmol was used as substrate the mannitol solution was infused at a rate of 9.5 ml/hr because harmol sulfate precipitates in the kidney and/or bladder at low urine flow rates, thereby blocking urine flow. At this high infusion rate the urine flow was 7 ml/hr. Bile duct and urine bladder were catheterized for continuous collection of bile and urine.

The phenols were injected intravenously in the femoral vein, they were dissolved in 1 ml aqueous 0.9 per cent (w/v) NaCl; 4-*t*-butylphenol was dissolved in saline by addition of sodium hydroxide to a final pH of 10.5 before dilution to the required concentration with saline. Unlabeled harmol was used in the *in vivo* experiments. Of the labeled phenolic compounds 7 μCi/kg was given.

In some experiments harmol was infused at a constant rate together with D-mannitol in the jugular vein to reach a steady-state in conjugation. The infusion rate of harmol was varied by changing the concentration of harmol in the D-mannitol (constant at 75 mg/ml) solution. The conjugates of harmol were determined in bile and urine. When the excretion rate of harmol sulfate was constant with time and the recovery of harmol as its conjugates was constant (more than 80 per cent of the infused amount) steady-state was assumed to have been achieved.

Isolation of rat hepatocytes. Liver cells were isolated according to the procedure of Berry and Friend [23] as modified by Vonk *et al.* [24]. For the preparation of hepatocytes the liver was submitted to a single pass perfusion at 37° via the portal vein with a Ca^{2+} free Hanks medium containing 22.5 mM $NaHCO_3$, gassed with carbogen (95 per cent O_2 and 5 per cent CO_2). After 8 min the perfusion was continued in a recirculating fashion with Hanks medium containing 0.03 per cent (w/v) collagenase and 1.3 mM Ca^{2+}. The perfusion was stopped after 20 min. The tissue was gently disrupted and the cell suspension was filtered through a plastic sieve (pore size 1 mm) and subsequently through nylon filter (pore size 100 μm). The cells were washed twice with incubation medium, filtered once more through the nylon filter and washed again twice with incubation medium. They were stored at 0° in the standard incubation medium (Krebs-bicarbonate) containing 119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.13 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5.0 mM glucose and 25 mM $NaHCO_3$. No albumine was present in the medium to avoid protein-binding of the substrates. The medium was buffered to pH 7.4 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid). The maximum period of storage before use was 1 hr.

Viability tests. Trypan blue exclusion by the isolated hepatocytes was tested by a 2 min incubation with trypan blue (0.4 per cent of the dye in the standard incubation medium). The intracellular K^+ concentration was determined according to Vonk *et al.* [24]. Values of 120–140 mM were considered to be normal. The cells were assessed routinely for microscopical appearance and trypan blue exclusion; if this exclusion was less than 95 per cent the cells were washed to remove damaged cells until 95 per cent trypan blue exclusion was reached. The yield was 240–280 × 10^6 hepatocytes from the liver of rats of 300 g (liver weight 10–12 g).

Incubation procedure with isolated rat hepatocytes. In plastic tubes 10^6 hepatocytes were incubated in a final volume of 200 μl incubation buffer (Krebs-bicarbonate). This high concentration of hepatocytes was required to obtain quantities of conjugates high enough to be determined reliably at the lower concentrations of substrate used. Therefore, it was used throughout this study. The tubes were shaken in a Dubnoff metabolic incubator while the tubes were gassed with carbogen and the temperature was kept at 37°. After 20 min of preincubation the reaction was started by addition of the substrate in 20 μl incubation medium. The incubation was terminated after 60 min when complete conjugation was ensured (unless otherwise stated) by the addition of 200 μl methanol in experiments with harmol, or by transferring the incubation mixture to glass tubes which contained 2 ml diethyl ether in the experiments with

the other three phenols. After thorough mixing on a vortex mixer the tubes were centrifugated at 1200 *g* for 10 min and a sample (50 μl) of the methanol extract or the ether-extracted aqueous layer was applied on thin layer chromatography. Extraction of the ^{14}C-labeled phenols in ether was more than 98 per cent while no detectable amounts of the conjugates were extracted.

Incubation of the hepatocytes during 60 min in the presence of 1200 μM phenol, 800 μM 4-chlorophenol, 800 μM 4-*t*-butylphenol or 300 μM harmol or in incubation buffer alone did not affect intracellular K^+ concentration.

Separation and quantitation of conjugates. Conjugates in bile, urine and hepatocyte incubations were separated by thin layer chromatography; 10 μl of samples of bile and urine or 50 μl samples of the hepatocyte incubation extracts prepared as described above were applied to the thin layer chromatography plates. Harmol conjugates were separated by the method of Mulder and Hagedoorn [20]; for the other phenols the method of Weitering *et al.* [4] proved to be suitable (see results).

The conjugates of unlabeled harmol were quantitated fluorometrically after thin layer chromatography by the method of Mulder and Hagedoorn [20]. In experiments with isolated hepatocytes ^{3}H-labeled harmol was used; in this case the radioactive spots on the thin layer chromatography-plates were scraped off and put in counting vials together with 0.5 ml water. After mixing 5 ml Picofluor was added for liquid scintillation counting. The same method was used for determination of the conjugates of the ^{14}C-labeled phenols.

Identification of conjugates. This was done as described by Weitering *et al.* [4].

RESULTS

Identification of conjugates

Samples of bile and urine of rats which had received [^{14}C]-4-chlorophenol or [^{14}C]-4-*t*-butylphenol were applied to thin layer chromatography plates. After chromatography the position of radioactive spots was determined by scraping off the plates in 2 cm zones and counting these for radioactivity.

The R_f values found were 0.0 and 0.48 (range 0.35–0.61) for 4-chlorophenol and 0.0 and 0.42 (range 0.23–0.61) for 4-*t*-butylphenol. The radioactivity of these spots disappeared completely after incubating bile and urine samples with a β-glucuronidase/arylsulfatase mixture, and was subsequently found at an R_f of 0.71, which is the R_f value of both unconjugated phenols. Incubation of bile and urine samples with these enzymes in the presence of the β-glucuronidase inhibitor D-glucaro-1,4-lactone resulted in the complete disappearance of the radioactivity from the spots with R_f values of 0.48 and 0.42 respectively; the radioactivity at the origin was not affected. Therefore, the radioactivity at the origin of the thin layer chromatography plates was the glucuronide conjugate for both phenols, and that at an R_f value of 0.48 for 4-chlorophenol and 0.42 for 4-*t*-butylphenol was the corresponding sulfate conjugate. Other radioactive spots were not found, and the radioactivity of the spots accounted for the total radioactivity applied to the thin layer chromatography plates. Therefore, the sulfate and glucuronide conjugates were the only metabolites excreted in bile and urine for both phenols.

Similar results were found when isolated hepatocytes were incubated with phenol, 4-chlorophenol, 4-*t*-butylphenol and harmol: glucuronide and sulfate conjugates were the only metabolites formed.

Table 1. Glucuronidation and sulfation of phenol, 4-chlorophenol, 4-*t*-butylphenol and harmol at increasing dose in the rat *in vivo*

Substrate	Dose (μmol/kg)	Glucuronide (per cent of dose)	Sulfate (per cent of dose)	Recovery (per cent of dose)
4-Chlorophenol	14	61 ± 3†‡	39 ± 3‖	100 ± 4
	26	63 ± 5§	39 ± 3¶	101 ± 3
	69	71 ± 7‡	32 ± 10	102 ± 3
	135	72 ± 3†§	29 ± 3‖¶	102 ± 3
4-*t*-Butylphenol	8	68 ± 7	21 ± 8	90 ± 1
	15	65 ± 4	29 ± 4†	93 ± 8
	28	71 ± 3	17 ± 3†‡	91 ± 7
	69	67 ± 3	29 ± 4‡	91 ± 3
Harmol	27	19 ± 1†‡§	81 ± 1‖¶**	100 ± 12
	67	31 ± 2†‡§	70 ± 2‖¶**	100 ± 4
	133	35 ± 2†‡§	56 ± 1‖¶**	90 ± 3
Phenol*	13	26	66	92
	27	33	63	96
	67	38	53	91
	133	47	47	93
	266	55	36	91

* Data taken from Ref. [4]. Rats were anesthesized with pentobarbital. Bile and urine were collected during 4 hr after the i.v. injection of the substrate. The amounts of conjugates excreted are given as percentage of the dose ± the S.D. For each dose 4 animals were used. In all experiments less than 1% was excreted as the unconjugated compound.

†, ‡, §, ‖, ¶, ** symbols denote significant differences ($P < 0.05$, Wilcoxon's test) between values marked with the same character within a substrate group.

Conjugation of various phenols in vivo

Since the *in vitro* determined K_m values for sulfation and glucuronidation [16] suggested that at increasing dose the sulfation of phenol, harmol and 4-chlorophenol would become saturated at a lower dose than glucuronidation while with 4-*t*-butylphenol the reverse was to be expected, we determined the conjugation patterns of these phenols by injecting them in the femoral vein at increasing doses. Bile and urine were collected during 4 hr thereafter and analyzed for the conjugation products. Table 1 gives the results. The recovery of the administered substrates was always between 90 and 100 per cent. The highest dose that could be given was limited for each phenol by its toxicity; higher doses resulted in most cases in the death of the animals within one minute after administration of the compound. It can be seen that sulfation of harmol decreased relative to its glucuronidation at increasing dose. For phenol the same results have been observed previously (Table 1, Ref. [4]). This shift was smaller, though still statistically significant, with 4-chlorophenol while it was completely absent with 4-*t*-butylphenol. The differences in the sulfation of 4-*t*-butylphenol at different doses did not show a consistent pattern and were caused only by the low sulfation at 28 μmol 4-*t*-butylphenol/kg. Both 4-chlorophenol and 4-*t*-butylphenol were mainly glucuronidated over the whole dose range tested, while harmol was mainly sulfated at all doses used.

After a single injection of a phenol *in vivo* the substrate supply to the transferases decreases with time. This complicates the interpretation of these *in vivo* results because the ratio between sulfation and glucuronidation may change in parallel continuously during the experiment. Therefore, for one substrate, harmol, we have tried to achieve a steady-state by constant intravenous infusion, to obtain a constant substrate supply.

Figure 1 shows the time-course of the excretion of the harmol conjugates in bile and urine, both at a low [0.05 μmol/min; Fig. 1(A)] and a high [2 μmol/min; Fig. 1(B)] infusion rate of harmol. Steady-state was reached after 80–120 min. However, at the higher infusion rate the steady-state was not yet reached for harmol glucuronide after 150 min. Variations in urine production during the experiments were the main cause of scatter of the data. Table 2 gives the results of a number of steady-state experiments relating steady-state excretion rates of harmol conjugates to the infusion rate of harmol. Clearly harmol excretion decreased rela-

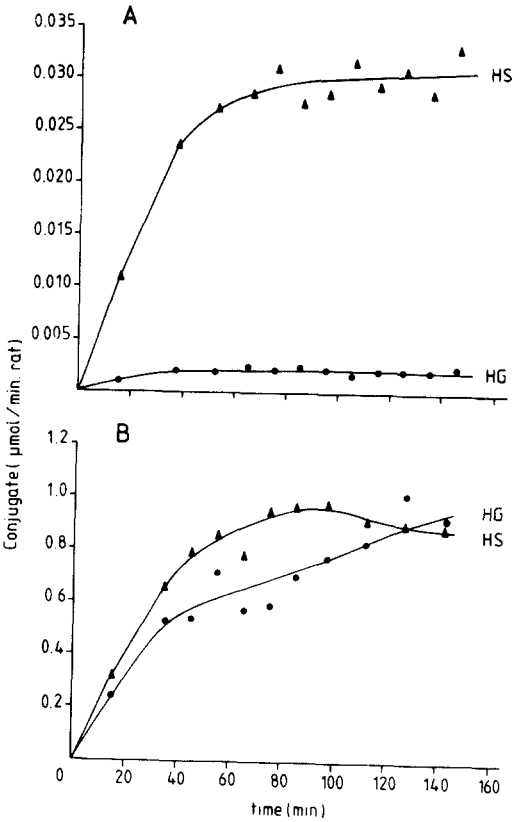

Fig. 1. Excretion rates of harmol sulfate (HS) and harmol glucuronide (HG) during i.v. infusion of harmol. Harmol was infused at various rates in the jugular vein in the same solution as D-mannitol (75 mg/ml) in 0.9 per cent (w/v) aqueous NaCl at a rate of 9.5 ml/hr. Bile and urine were collected in 10 min-fractions and analyzed for the conjugates excreted. The rates of excretion of the conjugates are plotted against time. These are typical examples. (A) Harmol infusion rate 0.05 μmol/min. (B) Harmol infusion rate 2.0 μmol/min.

Table 2. Conjugation of harmol *in vivo* in steady-state at various constant infusion rates

Infusion rate (μmol/min)	*n*	Recovery (per cent of infusion rate)	Harmol sulfate (μmol/min)	Harmol glucuronide (μmol/min)
0.05	3	88 ± 8	0.039 ± 0.002	0.005 ± 0.002
0.1	4	96 ± 11	0.069 ± 0.006	0.027 ± 0.006
0.5	3	91 ± 4	0.345 ± 0.002	0.10 ± 0.02
1.0	4	94 ± 9	0.64 ± 0.06	0.31 ± 0.04
1.5	4	90 ± 8	0.76 ± 0.05	0.59 ± 0.10
2.0	3	84 ± 6	0.77 ± 0.09	0.92 ± 0.04

Rats were anesthesized with pentobarbital. Harmol was infused in the jugular vein in the same solution as D-mannitol which was given as a solution of 75 mg/ml 0.9 per cent (w/v) aqueous NaCl at a rate of 9.5 ml/hr. The harmol infusion rate was varied by changing the harmol concentration in the D-mannitol solution. Bile and urine were collected and analyzed for the conjugates. The rates of excretion of the conjugates during steady-state are given with the standard deviations; *n* is the number of animals used.

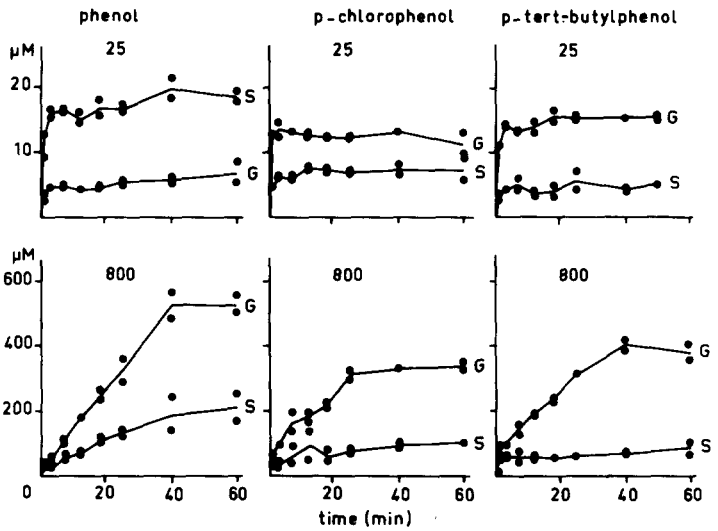

Fig. 2. Time course of conjugation of various phenols in isolated hepatocytes. Isolated rat hepatocytes were incubated with phenol (A), 4-chlorophenol (B) and 4-*t*-butylphenol (C) at 25 and 800 μM initially. Incubations lasted for 1 hr at 37°. The conjugates were separated by t.l.c. and quantitated by liquid scintillation counting. The amounts of the conjugates formed are expressed as the total concentrations in the incubation medium + cells: G = glucuronide; S = sulfate conjugate.

tive to harmol glucuronide excretion when the infusion rate was increased. However, sulfation was not severely affected by an eventual depletion of inorganic sulfate because the steady-state reached in harmol sulfation did not show a clear tendency to decrease during the experiments even at high infusion rates. Harmol glucuronide excretion increased even more than proportionally with increasing infusion rate of harmol. In this situation the shift is even more pronounced than in the single dose experiments where sulfation of harmol was predominant over the entire dose range.

Experiments with isolated rat hepatocytes

In vivo various organs besides the liver may contribute to the conjugation of phenols. To find out whether the *in vivo* observed dose-dependent shift from sulfation to glucuronidation also occurred during conjugation in liver cells alone, isolated hepatocytes were incubated with various concentrations of the phenols. The hepatocytes conjugated the substrates completely within a few minutes at the low concentration (25 μM) and within 40 min at a high initial concentration (800μM) (Fig. 2). A slower and incomplete conjugation was found at an initial concentration higher than 1200 μM for phenol, 800 μM for 4-chlorophenol and 4-*t*-butylphenol and 300 μM for harmol, probably due to toxic effect. The time-course of harmol conjugation is not shown; duplicate determination for sulfation in time showed wide variation, although the amounts of harmol sulfate synthesized at the end of the incubation, when all of the unconjugated harmol had been consumed, always were very close in spite of the variation in time course. We have no explanation for this phenomenon.

To investigate the conjugation pattern in relation to the substrate concentration, hepatocytes were incubated with a range of substrate concentrations, and the conjugates produced were analyzed (Fig. 3). In parallel with the results in the *in vivo* experiments, there was a marked difference in the concentration-dependent conjugation pattern of phenol and harmol on one hand and 4-chlorophenol and 4-*t*-butylphenol on the other. The former two substrates were mainly sulfated at low substrates concentrations while glucuronidation became more important when the concentration was increased. The latter two were mainly glucuronidated over the entire concentration range tested and no concentration-dependent shift in the ratio between sulfation and glucuronidation was observed.

DISCUSSION

The present data show that dose-dependent shifts of sulfation and glucuronidation of various phenols occur in parallel *in vivo* and in isolated hepatocytes. For two of these substrates, phenol and harmol, sulfation became limited at a lower substrate supply than glucuronidation.

The *in vivo* metabolism of a compound is influenced by many factors such as the presence of competing metabolic pathways, a limited availability of cosubstrates required in conjugation, saturation of the transferases or the uptake of the substrates by metabolizing organs, different contribution of various organs and first pass effects, determined by the route of administration.

The conjugation of phenol *in vivo* illustrates the importance of the route of administration [21, 22]: although high activities of phenol conjugating enzymes are present in the liver, phenol is extensively conjugated extra-hepatically by the lung during first pass through this organ, when the substrate is administered intraveneously. The dose used by Cassidy and Houston [21] was 15 μmol/kg which is about

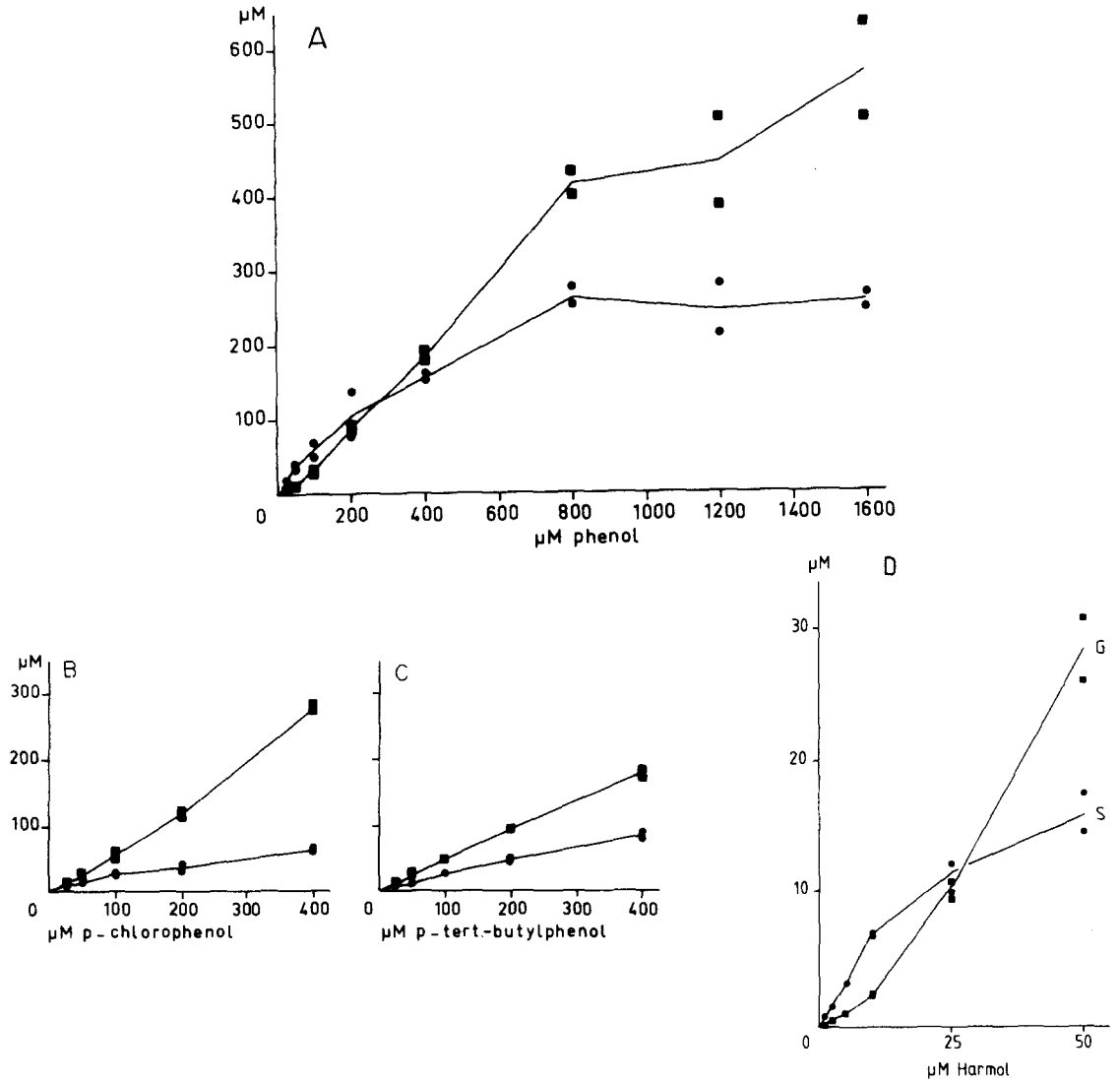

Fig. 3. Concentration dependence of the conjugation of various phenols. Phenol (A), 4-chlorophenol (B), 4-*t*-butylphenol (C) and harmol (D) were incubated for 1 hr at 37°. The conjugates were separated by t.l.c. and quantitated by liquid scintillation counting. The concentration of the conjugates represent the total concentrations in the incubation medium + cells: ■ = glucuronide; ● = sulfate conjugate.

the same as the lowest dose used by Weitering *et al.* [4] (Table 1). Of this dose 50–60 per cent is subject to first pass conjugation in the lung; this decreases to 30 per cent at a higher dose of 45 μmol/kg [21]. Presumably, the remainder of the dose is conjugated by the liver. If sulfation were the predominant conjugation in the lungs and glucuronidation that in the liver, a shift of conjugation from the lungs to the liver would result in a shift from sulfation to glucuronidation. Although such mechanism may operate *in vivo*, we found dose-dependent shift in the conjugation pattern for phenol and harmol in isolated hepatocytes that are similar to those observed with those phenol *in vivo*. Therefore, we conclude that conjugation in the liver alone would also show such a shift.

The possibility that depletion of inorganic sulfate occurs at high doses of substrate and, thereby would cause a decrease in sulfation has often been raised as a determining factor in the balance between sulfation and glucuronidation. In our experiments with isolated hepatocytes 1.2 mM inorganic sulfate was present in the incubation medium. The highest concentration of any sulfate conjugate formed was 250 μM (phenyl-sulfate, Fig. 3). This means that the amount of inorganic sulfate in the incubation medium was not decreased by more than 250 μM, or 16 per cent. Cheng and Levy [25] have shown that inorganic sulfate in the incubation medium rapidly equilibrates with inorganic sulfate in isolated hepatocytes, due to fast uptake of inorganic sulfate; the concentration of inorganic sulfate inside and outside the cells appears to be the same [25, 26]. Therefore, the rate of PAPS synthesis in the hepatocytes could not have been decreased by more than 16 per cent during the incubation. With harmol as substrate this decrease was only 2 per cent since at most 17 μM harmol sulfate was formed. If the availability of

inorganic sulfate would be the only limiting factor for sulfation in the cells, the relative decrease of sulfate conjugation should be no more than the decrease in availability, i.e. concentration of inorganic sulfate. However, the deviation of sulfation from linearity was found to be 47 per cent for phenol and 50 per cent for harmol (Fig. 3), which is much higher than the decrease in inorganic sulfate concentration can account for. This suggests that as the concentration of harmol or phenol increases, sulfation becomes saturated with the acceptor substrate rather than that sulfate or PAPS become depleted. Presumably, the same applies to the *in vivo* situation: The sulfation of a relatively high dose of phenol (266 μmol/kg) sulfation was only slightly increased by a high infusion of inorganic sulfate [16], indicating that sulfate-depletion did not play a role in the observed decrease of sulfation at this high dose. Furthermore, the amounts of inorganic sulfate required for sulfation of the other substrates used, could easily be provided *in vivo* [4, 16, 17, 27].

The most likely explanation of the dose-dependent shift of the ratio between sulfation and glucuronidation of phenol and harmol is the saturation of the sulfotransferase at a lower dose than UDP-glucuronyltransferase. This would imply a higher affinity for the sulfotransferase, as indeed was found *in vitro* for phenol, harmol and 4-chlorophenol [16, 20]. The latter substrate showed only a small, albeit significant, dose-dependent shift *in vivo* that was not seen in isolated hepatocytes. Although *in vitro* determined K_m values for conjugation of 4-*t*-butylphenol suggested that glucuronidation should decrease relative to sulfation as the dose was increased, yet, this substrate showed no dose-dependent shift at all in conjugation pattern, indicating that the *in vitro* determined kinetic parameters may be of doubtful significance *in vivo*. A pharmacokinetic approach may give more relevant information about the *in vivo* operating K_m values for sulfation and glucuronidation.

The fact that 4-chlorophenol and 4-*t*-butylphenol were mainly glucuronidated *in vivo* and in isolated hepatocytes might in some way be related to the fact that both these substrates showed (almost) no dose-dependent shift in sulfation and glucuronidation. Saturation of the sulfotransferase by these substrates may not have been reached at the doses used.

From the present findings we conclude that saturation of sulfation is more important in the balance between sulfation and glucuronidation *in vivo* at moderate doses of the substrate than a depletion of inorganic sulfate.

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REFERENCES

1. R. T. Williams, in *Biochemistry of Phenolic Compounds* (Ed. J. B. Harborne) p. 205. Academic Press, New York (1964).
2. I. D. Capel, R. T. Williams, M. R. French, P. Millburn and R. L. Smith, *Xenobiotica* **2**, 25 (1972).
3. R. T. Williams, *Biochem. J.* **32**, 878 (1938).
4. J. G. Weitering, K. R. Krijgsheld and G. J. Mulder, *Biochem. Pharmac.* **28**, 757 (1979).
5. R. Mehta, P. C. Hirom and P. Millburn, *Xenobiotica* **8**, 445 (1978).
6. H. G. Sammons, J. Shelswell and R. T. Williams, *Biochem. J.* **35**, 557 (1941).
7. D. J. Jollow, S. S. Thorgeirsson, W. C. Potter, M. Hashimoto and J. R. Mitchell, *Pharmacology* **12**, 251 (1974).
8. P. Eyer and G. Kampfmeyer, *Biochem. Pharmac.* **27**, 2223 (1978).
9. B. Anderson, M. Berggren and P. Moldéus, *Drug Metab. Disp.* **6**, 611 (1978).
10. P. Moldéus, *Biochem. Pharmac.* **27**, 2859 (1978).
11. P. Moldéus, H. Vadi and M. Berggren, *Acta Pharmac. Toxic.* **39**, 17 (1976).
12. P. Wiebkin, J. R. Fry, C. A. Jones, R. K. Lowing and J. W. Bridges, *Biochem. Pharmac.* **27**, 1899 (1978).
13. H. G. Bray, B. G. Hunphris, W. V. Thorpe, K. White and P. B. Wood, *Biochem. J.* **52**, 419 (1952).
14. K. P. Wong and T. Yeo, *Biochem. J.* **181**, 107 (1979).
15. G. J. Mulder and E. Scholtens, *Biochem. J.* **172**, 247 (1978).
16. G. J. Mulder and J. H. N. Meerman, in *Conjugation in Drug Transformation* (Ed. A. Aitio), p. 389. Elsevier/North Holland, Amsterdam (1978).
17. G. J. Mulder in *Sulfation of Drug and Related Compounds*, Chapter 6. CRC Press, Boca Raton, FL (1981).
18. G. J. Dutton, *Glucuronidation of Drugs and Other Compounds* CRC Press, Boca Raton, FL (1980).
19. W. B. Jakoby, R. D. Sekura, E. S. Lyon, C. J. Marcus and J-L Wang, *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby, J. R. Bend, J. Caldwell), Volume II. Academic Press, New York (1980).
20. G. J. Mulder and A. H. Hagedoorn, *Biochem. Pharmac.* **23**, 2101 (1974).
21. M. K. Cassidy and J. B. Houston, *Biochem. Pharmac.* **29**, 471 (1980).
22. M. K. Cassidy and J. B. Houston, *J. Pharm. Pharmac.* **32**, 57 (1980).
23. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
24. R. J. Vonk, P. A. Jekel, D. K. F. Meyer and M. J. Hardonk, *Biochem. Pharmac.* **27**, 397 (1978).
25. S. Cheng and D. Levy, *J. biol Chem.* **255**, 2637 (1980).
26. G. J. Mulder and K. Keulemans, *Biochem. J.* **176**, 959 (1978).
27. K. R. Krijgsheld, E. Scholtens and G. J. Mulder, *Comp. biochem. Physiol.* **67A**, 683 (1980).