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THE AVAILABILITY OF INORGANIC SULPHATE AS A RATE LIMITING FACTOR IN THE SULPHATE CONJUGATION OR XENOBIOTICS IN THE RAT?

SULPHATION AND GLUCURONIDATION OF PHENOL

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Abstract—Phenol is converted by the rat *in vivo* into the sulphate and glucuronide conjugates. When the intravenously administered dose of ^{14}C-labelled phenol was increased from 13 to 266 μmol/kg the percentage glucuronidated increased from 28 to 60 per cent of the dose; the percentage sulphated decreased from 72 to 40 per cent. In rats with intact kidneys the conjugates were almost completely excreted in the urine; when the kidneys were ligated phenylglucuronide was excreted in bile to a high extent, but biliary excretion of phenylsulphate was still very low. Using ^{35}S-labelled sodium sulphate, incorporation of [^{35}S]-sulphate into phenylsulphate could be observed; phenol enhanced the disappearance of [^{35}S]-sulphate from plasma. No significant depletion of inorganic sulphate was found when a high dose of unlabelled phenol (266 μmol/kg) was injected. The literature data on sulphate depletion by substrates of phenolsulphotransferase are critically reviewed; from our data is concluded that the relative decrease of phenol sulphation at high phenol doses is not due to sulphate depletion.

Many xenobiotics with a phenolic hydroxylgroup in their molecular structure are converted in mammals to both sulphate and glucuronide conjugates [1]. Therefore, the enzymes catalyzing these reactions, phenolsulphotransferase and UDP-glucuronyltransferase, compete for the same substrate when such a compound is given *in vivo*. Several authors have suggested that the pool of inorganic sulphate in the body may be severely depleted by sulphate conjugation when high doses of substrates of sulphotransferase are administered [2–5]. This assumption was used to explain the finding that often at high doses of phenolic substrates their sulphate conjugation becomes quantitatively less important, whereas the fraction that becomes glucuronidated increases. The alternative explanation, that the K_m of the sulphotransferase for the substrate may be much lower than the K_m for glucuronidation, has not been considered in most of these studies. In fact, the evidence for exhaustion of the sulphate pool is indirect and seems rather poor (see Discussion).

Since the relationship between dose dependence of the balance between glucuronidation and sulphation of a certain substrate, and a possible depletion of inorganic sulphate has not been studied extensively so far, we have started a detailed investigation on the conjugation *in vivo* of phenol in the rat. This substrate was chosen because it is almost completely converted into the sulphate and the glucuronide conjugate [6]. The data show that at increasing dose of phenol its glucuronidation increases relative to its sulphation.

In the discussion the literature data are critically reviewed; the conclusion is drawn from our results that plasma inorganic sulphate is not exhausted by phenol.

MATERIALS AND METHODS

Rats. Male Wistar rats (200 g body weight), were used throughout this study. They had free access to food and water. For the experiments they were anaesthesized with pentobarbital sodium (60 mg/kg intraperitoneally); they were kept at a body temperature between 37.5 and 38.5°. Artificial respiration was applied through a tracheal cannula, a bile duct cannula was inserted and the vena jugularis externa was cannulated. Through the latter cannula an intravenous infusion of mannitol, dissolved in aq. 0.9% (v/v) NaCl was given to ensure sufficient urine flow (see Results). The bladder was cannulated to collect urine continuously. Blood samples were taken through a cannula in the carotid artery; blood was collected in heparinized micro-tubes (Caraway Micro Blood Collecting Tubes, Sherwood Medical Industries Inc., St. Louis, MO, U.S.A.). Plasma was obtained by centrifugation of these tubes. Intravenous injections were given in the femoral vein in a volume of 2.5 ml of the solution per kg bodyweight. The blood pressure of some rats was monitored during the experiment. The intravenous dose of phenol varied between 13 and 266 μmol/kg bodyweight; when ^{14}C-labelled phenol was used about 7 μCi/kg was injected. When ^{35}S-labelled sodium sulphate was injected 50 μCi/kg was given intravenously, corresponding to about 0.1 μmol sodium sulphate per kg bodyweight. Bile and urine were collected in fractions of 10 min after injection of phenol or sodium sulphate.

Materials. ^{14}C-labelled phenol was obtained from New England Nuclear Inc., Dreieichenhain, Western Germany (specific radioactivity 10 mCi/mmol; dissolved in benzene). A stock solution for injection was prepared by extraction of phenol from the benzene layer into aq. 0.9% (v/v) NaCl; this solution was diluted with a solution of cold phenol to the appropriate concentration of phenol and radioactivity.

^{35}S-labelled sodium sulphate was also obtained from New England Nuclear Inc. as a solution in water (710 mCi/mmol); this stock solution was diluted be-

fore use with aq. 0.9% (v/v) NaCl. Mannitol was from Merck A.G., Darmstadt, Western Germany. The β-glucuronidase/arylsulphatase preparation was obtained from Boehringer Corp., Mannheim, Western Germany, and glucaro-1,4-lactone from Calbiochem, San Diego, CA, U.S.A. As scintillation medium for counting ^{14}C and ^{35}S, Plasmasol (New England Nuclear Inc.) was used.

Separation of phenol and its conjugates. Phenol and its metabolites were separated by thin layer chromatography. A sample of urine or bile was applied to a silicagel t.l.c. plate (Merck A.G.; catalogue number 5715). The plate was developed with butanol-1/water/ammonia (spec. grav. 0.91) 10:1:1 (v/v) [7]. After chromatography the spots were scraped off from the plate directly into a scintillation vial. After addition of Plasmasol they were counted in a liquid scintillation spectrometer. Usually 5–10 μl of urine or bile was applied to the plate. Plasma samples were first treated on ice with three volumes of methanol to precipitate the plasma proteins; radioactivity was completely retained in the supernatant. Then 50 μl of the methanolic supernatant was applied to the t.l.c. plate.

The main metabolites of phenol in the rat and many other mammalian species are phenylsulphate and phenylglucuronide [7]. After injection into a rat of ^{14}C-labelled phenol (266 μmol/kg i.v.; 7 μCi/kg), we have separated in our t.l.c. system three radioactive spots in a sample of urine of the rat. One of these, with a R_f value of 0.95, was identical with unconjugated phenol. It was present only at extremely low levels in urine obtained during the first few minutes after injection of [^{14}C]-phenol. However, when the t.l.c. plate was blown dry, or even when the plate was dried in air, phenol disappeared from the plate due to volatilisation; its spot should be scraped off from the plate when the silicagel is still saturated with the solvent.

The radioactivity at R_f 0.40 disappeared completely when the urine sample was incubated with an arylsulphatase preparation, before chromatography; the radioactivity was recovered at the phenol spot. The third radioactive spot was at the site of application of the urine sample (R_f of 0.0). This radioactivity could be displaced to the phenol spot when the urine sample was incubated with β-glucuronidase before chromatography; this hydrolysis was completely prevented by the presence of the specific β-glucuronidase inhibitor glucaro-1,4-lactone. Therefore we conclude that this is the glucuronide conjugate of phenol.

Mannitol-induced diuresis. In order to ensure a continuous and sufficient urine flow for collection of urine in fractions of 5–10 min from the bladder we have administered routinely an infusion in the vena jugularis externa of a mannitol solution (100 mg/ml water; infusion rate was 1.9 ml/hr after a priming dose of 50 mg mannitol. The body weight of the rats was 200 g). Fig. 1 shows a typical example of the response of a rat to a mannitol infusion at various infusion rates; its urine production can be increased tremendously over control, owing to the osmotic diuretic action of mannitol [10]. For our experiments we have used the relatively low infusion rate of 190 mg mannitol per hour, because that gave sufficient urine production during the two hours of the experiment. The volume of water infused was about equal to the volume of urine produced under these conditions.

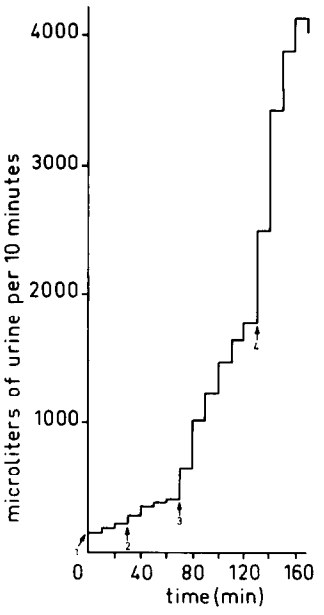

Fig. 1. Diuretic response of a rat on a mannitol infusion. An intravenous infusion of mannitol (75 mg of mannitol/ml water) was given to a rat at different infusion rates: 1. 1.9 ml/hr; 2. 3.8 ml/hr; 3. 9.9 ml/hr; and 4. 19 ml/hr. The infusion was preceded by a priming dose of 32.5 mg mannitol intravenously at $t = 0$. The bodyweight of the rat was 200 g.

Determination of inorganic sulphate in rat serum. Inorganic sulphate in rat serum was determined with a modification of the turbidimetric method of Berglund and Sörbo [9]. To 0.5 ml of serum (obtained in the absence of heparin) 2 ml of a 5% (w/v) trichloroacetic acid solution in water was added and the mixture was allowed to stand for 10 min at room temperature. After centrifugation 1 ml of the clear supernatant is added to 0.25 ml Barium chloride reagent (20 g $BaCl_2$ and 10 g dextran per litre water) and the absorbance was read after 35 min at 360 nm against a reagent blank (1 ml supernatant treated as above, but in the absence of $BaCl_2$). When inorganic sulphate is varied this results in a straight line (Frankena and Zweens, manuscript in preparation).

Separation of ^{35}S-labelled inorganic sulphate and phenylsulphate. Inorganic sulphate and phenylsulphate were separated by thin layer chromatography in the same system as described for the separation of phenol and its conjugates. ^{35}S-labelled inorganic sulphate remained completely at the site of application, whereas phenylsulphate had a R_f value of 0.35 in this system. The spots were scraped off from the plate, directly into a scintillation vial and counted for radioactivity as described before [8].

RESULTS

Dose dependent conjugation of phenol. Phenol was injected intravenously at five dose levels, 13–266 μmol/kg. Only at the highest dose a short lasting decrease of blood pressure was observed that recovered within five minutes. When this dose was given to conscious rats a tremor resulted that lasted about 2–5 min, after which the rats seemed to recover quickly and all rats survived this highest dose of phenol.

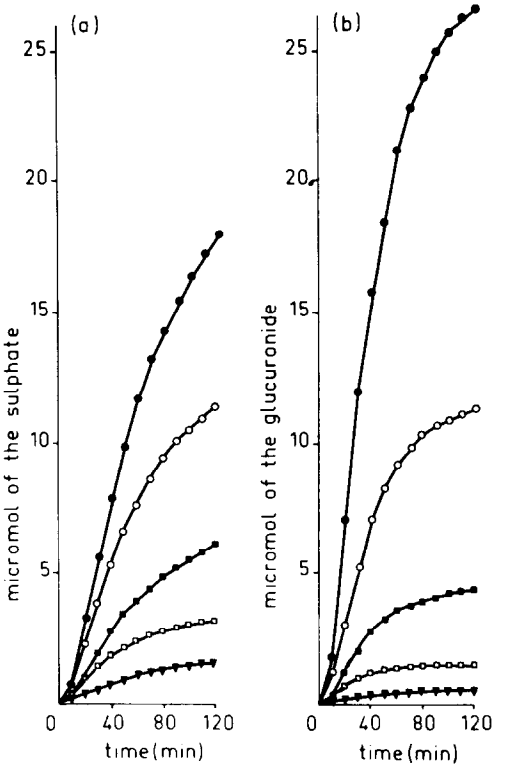

Fig. 2. Cumulative excretion in bile and urine of phenol conjugates after various intravenous doses of [^{14}C] phenol. ^{14}C-labelled phenol was injected intravenously into rats with a standard mannitol infusion at a dose of 13 (▼—▼), 27 (□—□), 66 (■—■), 133 (○—○) and 266 (●—●) μmol/kg. Samples of urine and bile were applied to t.l.c. for separation of the sulphate and glucuronide conjugates. These were determined by counting of radioactivity in the spots. For each dose of phenol the mean of the data of 2 rats is given cumulatively, expressed as μmol excreted as the sulphate (a) and glucuronide (b) conjugate.

Figure 2 shows the total amounts of phenylsulphate and phenylglucuronide excreted in bile and urine as a function of time after the injection of phenol at different doses; the amounts in bile and urine have been plotted cumulatively. Whereas at low doses the major part of phenol was converted to the sulphate conjugate, this was reversed at the highest dose, where the major part of the dose was glucuronidated. If the dose of phenol is doubled (Fig. 2) there was always more than twice as much glucuronide conjugate excreted, and always less than twice the amount of phenylsulphate. Yet, even at the highest dose of phenol, sulphation seems not yet saturated. Table 1 summarizes the data on the ratio between glucuronidation and sulphation of phenol. At about 133 μmol/kg phenol, equal amounts of both conjugates were formed.

Urinary and biliary excretion of the phenol conjugates; effect of ligation of the kidneys. Phenylsulphate was almost exclusively excreted in urine; only a trace of this conjugate could be found in bile, and then only in the first bile fraction after injection of phenol, at the highest dose. Most of the glucuronide was also excreted in urine but there was appreciable biliary excretion, especially at the higher doses. The time course of the excretion of phenylglucuronide in bile was different from that in urine: after an initial rapid biliary excretion during the first 40 min after injection of phenol, there was only very limited additional excretion in bile, whereas the urinary excretion still continued (Fig. 3).

In some experiments the kidneys were ligated and biliary excretion of the phenol conjugates was followed after a dose of 266 μmol/kg phenol intravenously. The amount of phenylglucuronide in bile was increased from 3.1 μmol per 2 hr in controls with intact kidneys to 9.0 μmol in rats with ligated kidneys. Therefore, for phenylglucuronide, the biliary excretion is an alternative for the urinary excretion when the kidneys are ligated. In the same period of 2 hr about 0.4 μmol of phenylsulphate was excreted in bile in rats with ligated kidneys whereas in controls less than 0.03 μmol was excreted; in the same period of time, however, about 18 μmol of phenylsulphate was excreted in urine in rats with intact kidneys, showing the very slow rate of biliary excretion of phenylsulphate.

Effects of a mannitol infusion at a higher rate on elimination of phenylsulphate and phenylglucuronide. In order to determine the effect of the diuresis by mannitol on the urinary excretion of the phenol conjugates we have tested the effect of a higher rate of the mannitol infusion. Instead of the usual 190 mg mannitol/hr we infused in some rats 750 mg/hr; the urine production increased from 200–300 μl/10 min to 1500–1800 μl/10 min. However, the excretion rates of the phenol conjugates were unaffected.

Is inorganic sulphate rate limiting for the sulphation rate of phenol? We have shown previously that inor-

Table 1. Dose dependence of sulphation and glucuronidation of phenol in the rat *in vivo*

Dose of phenol μmol/kg	Percentage excreted in bile and urine as phenylsulphate	phenylglucuronide	Percentage recovered
13	72	28	92
27	66	34	96
67	58	42	91
133	50	50	93
266	40	60	91

^{14}C-labelled phenol was injected intravenously in the femoral vein. Urine and bile were collected for 2 hours afterwards while the rat was under pentobarbital (Nembutal) anaesthesia. Urine flow was enhanced by a mannitol infusion in the vena jugularis externa (see Methods). The sulphate and glucuronide conjugates were separated by t.l.c. and determined by counting the radioactivity of the [^{14}C]-phenol in the conjugate. The results are the means of 2 rats at each dose.

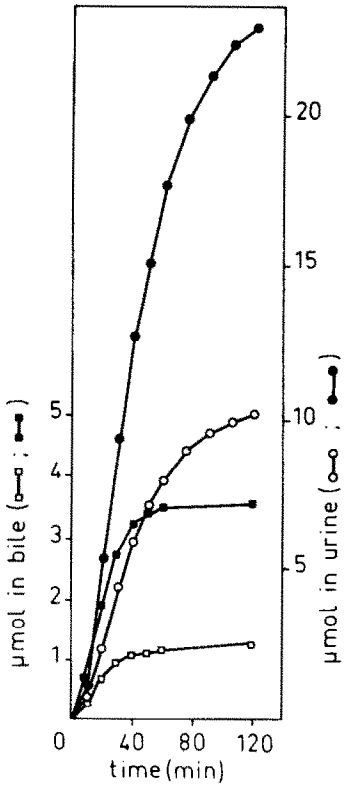

Fig. 3. Cumulative excretion of phenylglucuronide in urine and bile. The cumulative excretion of phenylglucuronide in bile after an intravenous injection of 266 μmol/kg (■—■) or 133 μmol/kg (□—□) of [^{14}C] phenol, and in urine after the same doses (respectively ●—● and ○—○) is plotted against time after injection. The conjugate was determined by counting the radioactivity of [^{14}C]phenol in the t.l.c. spots of the glucuronide conjugate

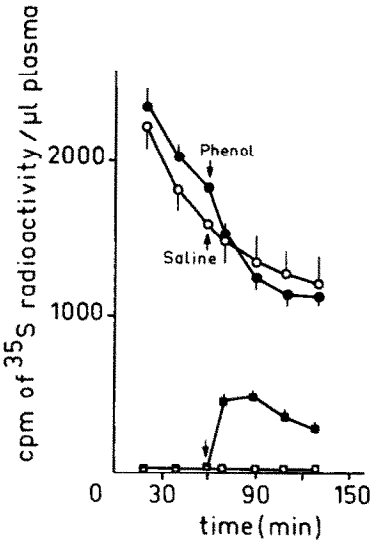

Fig. 4. Effect of an intravenous injection of phenol on disappearance of inorganic sulphate from plasma. ^{35}S-labelled sodium sulphate (200 μCi/kg) was injected intravenously at $t = 0$. Subsequently samples of blood were taken and the radioactivity in plasma was separated by t.l.c. into radioactivity at the site of application (●—●; ○—○) and at the phenylsulphate spot (■—■; □—□). The figure shows the radioactivity at these spots as a function of time after injection. At $t = 60$, after taking a sample of blood, either 0.9% (w/v) sodium chloride in water ('Saline'; ○—○; □—□) or phenol (266 μmol/kg; ●—●; ■—■) was injected intravenously. The results are the mean ± S.E.M. of 5 rats in each group.

ganic sulphate in plasma is immediately available for the sulphation of harmol [8]. To show that phenol uses the sulphate pool in blood for sulphation in the liver we have determined the effect of a dose of phenol on the plasma disappearance of ^{35}S-labelled sodium sulphate (Fig. 4). Inorganic [^{35}S]-sulphate in plasma disappeared at an enhanced rate after injection of phenol: from $t = 60$ min to $t = 90$ min (see Fig. 4) the radioactivity of inorganic sulphate decreased from 100 per cent (at $t = 60$ min) to 83.4 ± 4.5 per cent (mean ± S.E.M.) in controls that received no phenol, whereas in the rats that received phenol (266 μmol/kg) at $t = 60$ min the radioactivity in inorganic sulphate decreased from 100 per cent (at $t = 60$ min) to 67.8 ± 2.0 per cent at $t = 90$ min. The difference between controls and phenol-treated rats is statistically significant ($P < 0.01$; Wilcoxon's test). After injection of un-labelled phenol at $t = 60$ min (Fig. 4) radioactivity was found at the phenylsulphate spot, when a plasma sample was chromatographed, indicating incorporation of inorganic sulphate from plasma into phenylsulphate. In urine a great amount of radioactivity was found at the phenylsulphate spot, confirming the incorporation of ^{35}S-labelled sulphate into phenylsulphate.

These results show that plasma inorganic sulphate is required for sulphation of phenol. However, the plasma concentration of sulphate decreased only to half its original concentration (Fig. 4), and therefore much inorganic sulphate is still left in plasma.

Since it has been suggested by several authors [2–5] that the availability of inorganic sulphate may be rate limiting in the sulphation of drugs, we have done an experiment to find out if depletion of sulphate might explain why at increasing dose of phenol, glucuronidation rate increased more than sulphation rate. We have pretreated rats with an intravenous dose of unlabelled phenol (266 μmol/kg). If depletion of inorganic sulphate would play a role, a subsequent injection of phenol would show a decreased sulphation rate of the substrate. Therefore we injected, 60 min after the first dose of unlabelled phenol, a second dose of 67 μmol/kg of ^{14}C-labelled phenol intravenously. The percentage of the second dose that became sulphated was completely unaffected by the pretreatment with phenol (Table 2). Therefore, these data do not support the suggestion that inorganic sulphate had been depleted to any serious extent by the injection of the high dose of phenol.

These results were confirmed by measurements of serum inorganic sulphate concentration in rats after the injection of phenol (266 μmol/kg). Blood was collected from the aorta at different times after injection of phenol. The results (Table 3) show that although there was a statistically significant reduction in the plasma concentration of inorganic sulphate, this amounted to only about 25 per cent at most, and after 2 hr the plasma concentration was rising again. The implications of this will be considered in the Discussion.

Table 2. Lack of depletion of inorganic sulphate by a high dose of phenol in the rat *in vivo*

Conjugate in urine	μmol in urine during 2 hr: Without preceding phenol injection	After preceding phenol injection
Phenyl glucuronide	5.31 ± 0.28	5.85 ± 0.23
Phenyl sulphate	6.11 ± 0.24	6.00 ± 0.16

One group of rats received an intravenous dose of phenol (266 μmol/kg) during pentobarbital anaesthesia; a control group received only the solvent. One hour later a second injection was given intravenously of 67 μmol/kg ^{14}C-labelled phenol in both groups. The urine was collected during two hours (using the standard mannitol infusion) and the amounts of conjugates of phenol were determined. The means ± S.E.M. are given; the number of rats in each group was 5. The phenol pretreatment caused no statistically significant differences.

The serum concentration of inorganic sulphate in the rat is much higher than that in man, where it is about 0.2 mM as determined with the same method (Zweens and Frankena, personal communication).

DISCUSSION

Our results show that the ratio of sulphate to glucuronide conjugate formed from intravenously injected phenol is dose-dependent, decreasing at higher doses of phenol. It has often been suggested that sulphate conjugation of phenols and other substrates of sulphotransferases is limited by the availability of inorganic sulphate. Thus, Slotkin *et al.* [2] have claimed that sodium sulphite, co-administered with harmol, increased the ratio of sulphation to glucuronidation of harmol in the rat *in vivo*, as compared with controls that did not receive additional sulphite. Their own results, however, show that the increase of this ratio is not due to an increase of sulphation, but to a decrease of glucuronidation caused by sulphite pretreatment.

Büch *et al.* [4] have given huge intravenous doses of sodium sulphate to rats, increasing the plasma concentration of inorganic sulphate from about 0.5–0.8 mM [11] to probably 10–15 mM. Recently we have reported that inorganic sulphate in plasma is immediately available for sulphation of drugs in the liver [8]; moreover, the sulphate concentration, available for sulphation, in the liver is the same as the plasma concentration [20]. Therefore, the increase in sulphation of paracetamol *in vivo* that Büch *et al.* [4] have measured may well be due to the high concentration of inorganic sulphate as substrate for the synthesis of the cosubstrate of sulphation, adenosine 3′-phosphate 5′-sulphatophosphate. The few reported K_m values for sulphate are of the order of 0.1–5 mM [12–15].

Table 3. Effect of a high dose of phenol on plasma concentration of inorganic sulphate in the rat

Time after injection of phenol (min)	Concentration of inorganic sulphate in serum (mM; mean ± S.E.M.)
Control	0.92 ± 0.03
5	0.79 ± 0.05*
10	0.86 ± 0.02
15	0.73 + 0.05*
20	0.77 ± 0.05†
30	0.72 ± 0.02†
60	0.71 ± 0.04†
120	0.83 ± 0.04†‡

Phenol (266 μmol/kg) was injected intravenously into the tail vein in conscious rats. After the time indicated blood was collected from the aorta with a syringe; the rats received an ether anaesthesia. Serum was obtained and inorganic sulphate was determined as described in the Methods. The number of animals in each group was 6, but in the control group were 8 rats. * Means significantly different from control at $P < 0.025$; †the same at $P < 0.005$. ‡Means significantly different from the data at $t = 60$ at $P < 0.05$.

Our experiments show that exhaustion of the sulphate pool by phenol is an unlikely explanation for the relative decrease in sulphation at higher doses of phenol. Although the high demand of inorganic sulphate at the highest dose of phenol (Fig. 4) caused a rapid initial decrease of ^{35}S-labelled sulphate in plasma, this decrease could be compensated fairly rapidly by (presumably) an increased supply from other sites in the body: a second injection of phenol, one hour after the first, showed the same conjugation pattern as if no phenol had been injected before (Table 2). If we assume that the extracellular space is 20 per cent of body volume, then, at a plasma concentration of 0.9 mM in the rat, about 180 μmol/kg of extracellular sulphate would be immediately available (in the mouse the sulphate pool is 150–200 μmol/kg [16]). About 105 μmol/kg was required for the sulphate conjugation of 266 μmol/kg phenol (Table 1); this would leave only 70 μmol/kg extracellular sulphate available. Yet even the rate of sulphation of a second dose of phenol, requiring about 50 μmol/kg of sulphate, was unaffected (these are the detailed results from which Table 2 is composed), indicating that the loss of inorganic sulphate had been compensated by replenishment from other sites. In that case the rat would be able to mobilize considerable amounts of sulphate, in agreement with the data of Büch *et al.* [4] who found that the urinary output of inorganic sulphate is about 45–60 μmol/kg/hr (female rats). This is also confirmed by our data on the serum concentration of inorganic sulphate, that was decreased only little by the phenol injection. If only extracellular inorganic sulphate were used it should have decreased to about 0.4 mM, whereas its lowest

point was still about 0.7 mM (Table 3). This is further confirmed by the data of Fig. 4 that show that the phenol injection caused only a relatively small decrease of the [^{35}S]-sulphate concentration in plasma. Thus, the rat is able to cope with a high demand of sulphate, even when a high dose of phenol is given.

When increasing the dose of phenol from 13 to 26 μmol/kg, requiring only about 10 and 18 μmol/kg of inorganic sulphate for conjugation respectively, we already observed a relative decrease in sulphation. From the above discussion it seems unlikely that in this case depletion of sulphate in blood would be the cause of the relative decrease of sulphation. Since sulphate in plasma is immediately available for sulphation in the liver [8] it is also unlikely that the sulphate supply specifically in the liver might be rate limiting.

An alternative explanation for the relative decrease of sulphation at higher dose of phenol may be that the K_m for sulphation is much lower than for glucuronidation. At lower doses of phenol, in that case, the competition between sulphation and glucuronidation for the same substrate would favour sulphate conjugation. At increasing dose the rate of glucuronidation would increase more than the rate of sulphate conjugation; at a certain dose the latter would be saturated and only glucuronidation would increase further. Capel *et al.* [17] also suggested that such a difference in K_m might be one explanation of their findings with phenol in the hen. It might explain the results of Minck *et al.* [18] who found mainly sulphation of *p*-nitrophenol when this compound was generated in the liver by demethylation of *p*-nitroanisol, whereas *p*-nitrophenol itself was mainly glucuronidated. This may be due to differences in the substrate concentration of *p*-nitrophenol in the liver when it was generated *in situ* being low, and being high when it was presented as such.

The present results confirm the predominant urinary excretion of the phenol conjugates, in agreement with the observation that compounds with a molecular weight below 325 are little excreted in bile [19]. If, however, the urinary excretion is impossible, e.g. during ligation of the kidneys, we find that biliary excretion does occur, be it at a slower rate than urinary excretion.

In conclusion, the decrease of sulphation of phenol at increasing dose can not be explained by a depletion of inorganic sulphate. It remains to be seen, whether it can be explained solely by the difference in affinity of phenol for UDP-glucuronyltransferase and phenol-sulphotransferase.

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