Metabolism of Inorganic Sulphate in the Isolated Perfused Rat Liver

EFFECT OF SULPHATE CONCENTRATION ON THE RATE OF SULPHATION BY PHENOL SULPHOTRANSFERASE

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1. The metabolism of inorganic [^{35}S]sulphate ($\text{Na}_2^{35}\text{SO}_4$) was studied in the isolated perfused rat liver at three initial concentrations of inorganic sulphate in the perfusion medium (0, 0.65 and 1.30 mM), in relation to sulphation and glucuronidation of a phenolic drug, harmol (7-hydroxy-1-methyl-9H-pyrido[3,4-b]indole). 2. [^{35}S]Sulphate rapidly equilibrated with endogenous sulphate in the liver. It was excreted in bile and reached, at the lowest concentration in the perfusion medium, concentrations in bile that were much higher than those in the perfusion medium; at the higher sulphate concentrations, these concentrations were equal. The physiological concentration of inorganic sulphate in the liver, available for sulphation of drugs, is similar to the plasma concentration. 3. At zero initial inorganic sulphate in the perfusion medium, the rate of sulphation was very low and harmol was mainly glucuronidated. At 0.65 mM-sulphate glucuronidation was much decreased and considerable sulphation took place, indicating efficient competition of conjugation by sulphation. At 1.30 mM-sulphate the sulphation increased still further. 4. The results suggest that an important factor in sulphation is the relatively high K_m of synthesis of adenosine 3'-phosphate 5'-sulphatophosphate (the co-substrate of sulphation) for inorganic sulphate, which is of the order of the plasma concentration of inorganic sulphate. The steady-state adenosine 3'-phosphate 5'-sulphatophosphate concentration may determine the rate of sulphate conjugation of drugs in the rat *in vivo*.

Many drugs and endogenous substances are metabolized in mammals to sulphate conjugates. These usually are rapidly excreted in urine or bile (Millburn, 1970). The co-substrate required for sulphation, adenosine 3'-phosphate 5'-sulphatophosphate, is synthesized from ATP and inorganic sulphate in those tissues where sulphation takes place (Roy, 1971). For xenobiotics this occurs predominantly in the liver, but the kidneys and the intestinal mucosa may contribute considerably (Roy, 1971). In many tissues sulphate is also required for synthesis of sulphated macromolecules, such as those present in the Golgi system in the liver (Katona, 1976), or in cartilage (Herbai, 1970).

For these sulphation reactions the body requires a continuous supply of inorganic sulphate in order to synthesize the adenosine 3'-phosphate 5'-sulphatophosphate needed. Inorganic sulphate in blood can be provided by S-oxidation of cysteine, taken up as such or as methionine with the food; it is as yet unclear to what extent inorganic sulphate is taken up from the intestinal lumen. It has been suggested that the inorganic sulphate supply of the body can be

exhausted when drugs that are mainly sulphated are given in high doses. The evidence suggesting exhaustion of sulphate under these circumstances is, however, rather poor, as we have discussed elsewhere (Weitering & Mulder, 1978). An important factor seems to be the lack of a sensitive assay method for inorganic sulphate in plasma and tissues; thus the plasma concentration of inorganic sulphate had never been measured after a load of a substance that is mainly sulphated *in vivo*.

We have tried to ascertain further the role of the liver in sulphate metabolism by studying this in the isolated perfused rat liver. We were especially interested in sulphation and glucuronidation of a phenolic substrate, harmol (7-hydroxy-1-methyl-9H-pyrido[3,4-b]indole), in relation to availability and concentration of inorganic sulphate in the perfusion system; so far, no such studies are available. We have used $\text{Na}_2^{35}\text{SO}_4$ to follow the metabolism of inorganic sulphate. Our results suggest that a decreased steady-state concentration of adenosine 3'-phosphate 5'-sulphatophosphate, rather than exhaustion of inorganic sulphate as such, may be the main reason

for a decrease in the rate of sulphation of drugs when they are given at high doses.

Materials and Methods

Rats and perfusion procedure

Male Wistar rats (body weight 295 ± 6 g) were used as donors of the liver. The rats had free access to food and water. They were anaesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) and the liver was removed for perfusion as described by Meijer & Weitering (1970). The liver weight was 8.7 ± 0.6 g (mean $\pm$ S.E.M.). In all experiments 100 ml of erythrocyte-free perfusion medium was used, containing Krebs bicarbonate buffer (Umbreit *et al.*, 1957) from which sulphate was omitted; Na_2SO_4 was added to the solution as required to provide initial concentrations of 0.00, 0.65 and 1.30 mM inorganic sulphate in the perfusion medium. The perfusion medium contained also 1% (w/v) demineralized bovine serum albumin. In these experiments with erythrocyte-free perfusion medium, sufficient oxygen was provided by an enhanced perfusion rate of 35 ml/min. To replace the bile salts, normally re-entering from the enterohepatic circulation, in all experiments an infusion of $15 \mu\text{mol}$ of taurocholate/h was given, dissolved in aq. 0.9% (w/v) NaCl (0.9 ml/h). The viability of the liver was tested by measuring perfusion flow through the liver, the pH of the perfusion medium and the bile flow, during the 3 h of the perfusion. Bile flow amounted in control perfusions to about $12 \mu\text{l}/\text{min}$ and decreased only slightly towards the end of the perfusion.

About 30 min after initiation of the perfusion (during which period the liver equilibrated with the perfusion medium containing one of the three inorganic sulphate concentrations), $\text{Na}_2^{35}\text{SO}_4$ was added (the same amount in all perfusions: 12.5×10^6 c.p.m.). Exactly 10 min thereafter $16 \mu\text{mol}$ of harmol was added to the perfusion medium, resulting in an initial concentration of 0.16 mM in the perfusion medium. Samples of the perfusion medium were taken for analysis at various times, and bile was collected in 15 min fractions before and after the addition of harmol. In control perfusions harmol was added 120 min after the addition of $\text{Na}_2^{35}\text{SO}_4$; in these perfusions we could follow the behaviour of inorganic sulphate under conditions where no exogenous substrate for sulphation was present. The perfusion lasted for 3 h after the addition of harmol or, in controls, for 3 h after addition of $\text{Na}_2^{35}\text{SO}_4$. Subsequently the liver was homogenized with a Potter-Elvehjem homogenizer in 4 vol. of aq. 0.9% (w/v) NaCl; a sample was counted by liquid-scintillation spectrometry for radioactivity, after being dissolved in scintillator containing Plasmasol (New England Nuclear Inc., Dreieichenhain, W. Germany).

Materials

Harmol, $\text{Na}_2^{35}\text{SO}_4$ and other chemicals were from the sources reported before (Mulder & Hagedoorn, 1974; Mulder & Scholtens, 1978). Bovine serum albumin (demineralized) was obtained from Poviet Producten, Oss, The Netherlands, Taurocholate was from Calbiochem, San Diego, CA, U.S.A.

Separation and determination of harmol and its conjugates

Harmol and its glucuronide and sulphate conjugate were separated by t.l.c. as reported before; they were measured fluorimetrically (Mulder & Hagedoorn, 1974). Samples of bile (usually $10 \mu\text{l}$) and perfusion medium ($20 \mu\text{l}$; used after centrifugation of the sample for 20 min at $3000g_{av.}$) were applied to the plate without further purification or extraction.

Determination of ^{35}S in inorganic sulphate and in harmol sulphate

Inorganic sulphate and harmol sulphate were separated in the same t.l.c. system as that used above for separation of harmol and its conjugates (Mulder & Scholtens, 1978). Inorganic sulphate remained at the site of application of the sample, whereas harmol sulphate had R_F 0.35–0.45 in this system. After separation the spots were scraped from the plate directly into scintillation vials and counted for radioactivity by liquid-scintillation spectrometry after addition of Plasmasol (Mulder & Scholtens, 1978).

Results

Equilibration of inorganic sulphate in the perfusion medium with the endogenous sulphate pool in the isolated perfused rat liver

We perfused the isolated rat livers at three different concentrations of inorganic sulphate in the perfusion medium, 0, 0.65 and 1.30 mM (initially), dissolved in Krebs bicarbonate buffer from which sulphate was omitted. Inorganic sulphate originally present in the liver was, of course, also present in the system; we show below that this amount contributes only 0.03–0.04 mM to the sulphate concentration in the perfusion medium. The same amount of $\text{Na}_2^{35}\text{SO}_4$, 125000 c.p.m./ml of perfusion medium, was added to the perfusion at all concentrations of sulphate in the perfusion medium. Within 8 min after addition of the labelled sulphate the concentration of the radioactivity in the perfusion medium entering and leaving the liver was the same, indicating that after that time the uptake of ^{35}S sulphate by the liver was very low. During the first 8 min the liver did take up some radioactivity, especially in the perfusions without added sulphate (zero concentration); at the higher sulphate concentrations the uptake by the liver was too small to cause a decrease of radioactivity

Fig. 1. *Effect of harmol on the disappearance of [^{35}S]sulphate from the perfusion medium at 0mM-inorganic sulphate*
 The disappearance of [^{35}S]sulphate (125 000 c.p.m./ml of perfusion medium initially) was determined at 0mM-inorganic sulphate in the perfusion medium. At the times indicated 20 μl of the perfusion medium was taken and used for t.l.c.; inorganic sulphate and harmol sulphate were separated. The amount of radioactivity in the inorganic sulphate spot (Δ , $\bullet$) and the harmol sulphate spot ($\circ$) are given as c.p.m./20 μl of perfusion medium. Harmol was added either 10 min after $\text{Na}_2^{35}\text{SO}_4$, at $t = 0$ (Δ), or 130 min thereafter ($\bullet$, $\circ$; see the arrow). The experiment in which harmol was added at $t = 0$ shows the mean of four separate experiments; the control experiment (harmol added at $t = 130$) is the mean of two separate perfusions.

in the perfusion medium during passage through the liver.

During the 3 h of the perfusion there was virtually no decrease of radioactivity of inorganic sulphate in the perfusion medium at the higher concentrations of sulphate, even though a substrate of sulphation, harmol, was present (see below). At the lower concentration of sulphate (zero initially) only inorganic sulphate from the liver was available, and presumably it leaked out of the liver into the perfusion medium. The results show (Fig. 1) that in the control perfusion at 0mM-sulphate (in which harmol was added only after an initial period of 2 h), during the first 2 h only a slow decrease of inorganic sulphate was observed; a rapid fall in the concentration of sulphate in the perfusion medium was seen, however, when harmol was added to the perfusion, both immediately after addition of $\text{Na}_2^{35}\text{SO}_4$ and 2 h thereafter. This indicates enhanced utilization of sulphate. At 0mM-

sulphate, in the absence of harmol, about 15–20% of the initial [^{35}S]sulphate had disappeared from the perfusion medium 2 h after its addition.

Inorganic sulphate from the perfusion medium was excreted in bile. At the two higher concentrations of inorganic sulphate almost identical amounts of radioactivity in inorganic sulphate were found in bile, indicating that doubling the plasma concentration of sulphate resulted in doubling of its biliary excretion. Much more radioactivity was excreted at the lowest sulphate concentration, which is surprising, since it indicates that a much greater fraction of inorganic sulphate from the perfusion medium was excreted than at the higher sulphate concentrations. This is confirmed by the finding that at 0mM-sulphate the inorganic [^{35}S]sulphate concentration in bile was about 5–6-fold that in the perfusion medium, whereas at 1.30mM-sulphate these concentrations were equal (Fig. 2). In all cases the initial phase of the

biliary excretion was linear with time, indicating a rapid mixing of the [^{35}S]sulphate pool in the perfusion medium with the endogenous hepatic sulphate pool.

Fig. 2. Concentration of inorganic [^{35}S]sulphate in the perfusion medium and in bile

The concentrations of inorganic [^{35}S]sulphate in bile fractions collected during perfusion experiments at 0 and 1.30 mM-sulphate in the perfusion medium, and in the perfusion medium, are given as c.p.m./10 μl of bile ($\circ$, $\triangle$) or perfusion medium ($\bullet$, $\blacktriangle$). The radioactivity was determined after t.l.c. of samples of bile and perfusion medium; harmol was present in these perfusions from $t = 0$.

Only a minor proportion of the inorganic sulphate had been excreted as such in bile during the 3 h of the perfusion: 1–5% of the dose (Table 1).

Sulphation and glucuronidation of harmol as a function of the inorganic sulphate concentration in the perfusion medium

We have measured the conjugation pattern of harmol in the liver perfusion at the three sulphate concentrations studied. In agreement with our previous results (Mulder *et al.*, 1975), we found a rapid and complete disappearance of harmol from the perfusion medium within 15 min after its addition. Only traces of unconjugated harmol were found in bile, less than 2% of the dose after 3 h (Table 2). Most of the substrate was recovered as harmol sulphate and harmol glucuronide in bile (Fig. 3; Table 2). In Fig. 3(a) the biliary excretion of harmol glucuronide is plotted cumulatively; at the two higher concentrations of inorganic sulphate there is no statistically significant difference between the percentage of the dose converted into the glucuronide conjugate, but at the lowest sulphate concentration we found almost double the amount of harmol glucuronide. During the first 30 min, 80–90% of the amount of harmol glucuronide ultimately recovered in bile after 3 h had already been excreted in bile; for the sulphate conjugate the corresponding value was 30–40% (Fig. 3b), indicating a marked difference in excretion pattern between the two conjugates (Mulder & Scholtens, 1977). At 0.65 mM-sulphate in the perfusion medium, the sulphate conjugation of harmol increased considerably over that at 0 mM-sulphate (Fig. 3b): it was almost twice as much. When sulphate was further increased to 1.30 mM there was a further increase in the excretion of harmol sulphate, about 30%.

Table 1. Recovery of ^{35}S radioactivity after addition of $\text{Na}_2^{35}\text{SO}_4$ to the isolated perfused rat liver
Harmol (16 μmol) was added to the perfusion system at three different concentrations of inorganic sulphate, 10 min after addition of $\text{Na}_2^{35}\text{SO}_4$; the perfusion was terminated 3 h after the addition of $\text{Na}_2^{35}\text{SO}_4$. The amounts of radioactivity in the perfusion medium at that time, the amounts excreted in bile and the amounts present in the liver (determined after homogenization of the liver) are expressed as percentages of the amount of radioactivity added to the perfusion medium. Bile and plasma radioactivity were separated in inorganic sulphate and harmol sulphate by t.l.c. All results are the means $\pm$ S.E.M. and n is the number of perfusions at each concentration of inorganic sulphate. Initially 125000 c.p.m. was added/ml of perfusion medium.

Concn. of inorganic sulphate in perfusion medium (mM)	n	Radioactivity (% of added c.p.m.)					
		Excretion in bile during 3 h		In perfusion medium		Total radioactivity in liver	Total recovery
		Inorganic sulphate	Harmol sulphate	Inorganic sulphate	Harmol sulphate		
0.00	4	5.4 ± 0.6	24.7 ± 3.3	32.8 ± 2.3	5.3 ± 1.3	14.5 ± 1.4	83.7 ± 4.5
0.65	4	1.2 ± 0.1	1.9 ± 0.1	65.3 ± 4.0	2.1 ± 0.2	5.7 ± 0.5	76.2 ± 4.5
1.30	5	1.0 ± 0.1	1.3 ± 0.2	74.5 ± 3.8	1.9 ± 0.2	5.3 ± 0.3	84.0 ± 3.8

Table 2. Sulphation and glucuronidation of harmol at different concentrations of inorganic sulphate in the perfusion medium of the isolated perfused rat liver

Harmol (16 μ mol) was added to the perfusion medium of the isolated perfused rat liver at three concentrations of inorganic sulphate. The amounts of the harmol conjugates and of unchanged harmol excreted during the subsequent 3 h in bile were determined fluorimetrically; they are given as percentages of the dose of harmol (means $\pm$ S.E.M.). In the two control groups harmol was added 2 h after the addition of $\text{Na}_2^{35}\text{SO}_4$ (see the Materials and Methods section), at 0 and 1.30 mM-inorganic sulphate in the perfusion medium. Here bile was collected for only 1 h after addition of harmol; the mean for two experiments is given. n is the number of perfusions.

Concn. of inorganic sulphate in perfusion medium (mM)	n	Harmol glucuronide (%)	Harmol sulphate (%)	Unchanged harmol (%)
0.00	4	67.7 $\pm$ 4.6	7.5 $\pm$ 0.4	1.7 $\pm$ 0.2
0.65	4	43.7 $\pm$ 2.5	14.9 $\pm$ 1.7	1.4 $\pm$ 0.2
1.30	5	39.3 $\pm$ 2.5	19.5 $\pm$ 2.7	1.3 $\pm$ 0.4
Control at 0.00	2	59 (1 h)	5 (1 h)	0.2 (1 h)
Control at 1.30	2	33 (1 h)	7 (1 h)	0.4 (1 h)

Fig. 3. Biliary excretion of (a) harmol glucuronide and (b) harmol sulphate after addition of harmol to the perfusion at different concentrations of inorganic sulphate

The concentration of inorganic sulphate (initially) in the perfusion medium was 0 ($\bullet$), 0.65 ($\circ$) or 1.30 (Δ) mM. At 10 min after the addition of $\text{Na}_2^{35}\text{SO}_4$, harmol (16 μ mol) was added, at $t = 0$. A 10 μ l sample of bile was subjected to t.l.c. and harmol glucuronide and harmol sulphate were separated; they were determined fluorimetrically and the total amount of each in bile is given cumulatively for the glucuronide (a) and the sulphate (b) conjugates. The means $\pm$ S.E.M. (bar) are given for four ($\bullet$, $\circ$) or five (Δ) perfusions.

Incorporation of [^{35}S]sulphate into harmol sulphate

When harmol was added to the perfusion medium small amounts of ^{35}S radioactivity were found in harmol sulphate in the perfusion medium (Fig. 1), indicating incorporation of [^{35}S]sulphate into harmol sulphate. In bile large amounts of radioactivity were found in harmol sulphate, especially in the low-

sulphate perfusion: as much as 25% of the radioactivity was recovered in bile as harmol sulphate (Table 1). As expected, much less radioactivity was incorporated into harmol sulphate at the two higher sulphate concentrations (Table 1), since there the specific radioactivity of inorganic sulphate was much lower. The specific radioactivity of inorganic sulphate

was calculated from the amount of harmol sulphate (determined fluorimetrically) and the amount of radioactivity incorporated into harmol sulphate in every fraction of bile collected. We found 2070, 107 and 56 c.p.m./nmol of harmol sulphate respectively at 0, 0.65 and 1.30 mM-sulphate in the perfusion medium.

In control perfusions for 2 h (without harmol) at both 0 and 1.30 mM-sulphate virtually no radioactivity was found in bile at the harmol sulphate spot on t.l.c. When harmol was added 2 h after the addition of [^{35}S]sulphate in the control perfusion (Table 2), clearly [^{35}S]sulphate was incorporated into harmol sulphate; moreover, glucuronidation was the same as in the experiments in which harmol was added immediately after $\text{Na}_2^{35}\text{SO}_4$ (Table 2).

Discussion

Equilibration of sulphate in the perfusion medium with the endogenous sulphate pool in the liver

We have previously shown that a rapid equilibration of inorganic sulphate takes place between perfusion medium or plasma and the liver (Mulder & Scholtens, 1978). From the specific radioactivity of inorganic sulphate, as determined from harmol [^{35}S]sulphate, the amount of sulphate originally present in the liver can be calculated, if it is assumed that both pools are completely mixed. The specific radioactivity of the total inorganic sulphate pool in the perfusion system equals (eqn. 1) the amount of radioactivity added divided by the amount of sulphate in the perfusion, i.e. the sum of the sulphate added in the perfusion medium (Y) and that present in the liver originally (C). If the reciprocal of the specific radioactivity is plotted (eqn. 2) against the amount of sulphate in the perfusion medium (Y), the intersection with the abscissa gives the amount of

$$\text{Sp. radioactivity} = \frac{\text{radioactivity added}}{Y + C} \quad (1)$$

$$Y + C = \frac{1}{\text{Sp. radioactivity}} \times \text{radioactivity added} \quad (2)$$

sulphate originally present in the liver (C). Fig. 4 shows this plot; the endogenous pool is about 3–4 μmol . Since the mean weight of the livers used was 8.7 g, this implies an overall concentration of inorganic sulphate in the liver of about 0.4 mM; if it was present only in the cytosol this would be about 0.8 mM. The serum concentration of inorganic sulphate in our rats is 0.85 ± 0.04 mM (Weitering & Mulder, 1978).

Conjugation of harmol as a function of the concentration of inorganic sulphate in the perfusion medium

The results show that glucuronidation easily compensates for the loss of sulphation at the lowest

Fig. 4. Plot for determination of the hepatic endogenous sulphate pool

The reciprocal of the specific radioactivity of harmol sulphate is plotted against the amount of inorganic sulphate in the perfusion medium. For details see the Discussion section. The intersect with the abscissa equals C , the amount of inorganic sulphate available for sulphation of harmol in the liver. The mean weight of the livers used for this plot was 8.7 g.

concentration of inorganic sulphate in the perfusion medium. Therefore sulphation efficiently competes with glucuronidation, especially at low doses of harmol, presumably owing to a much higher affinity of phenol sulphotransferase for harmol than that of UDP-glucuronyltransferase (Mulder *et al.*, 1975; in those experiments the sulphate concentration in the perfusion medium was 1.30 mM). At the lowest concentration of sulphate only the 3–4 μmol of endogenous sulphate in the liver was present; assuming that this hepatic pool is rapidly redistributed over the perfusion medium and the liver, an overall sulphate concentration of about 0.03–0.04 mM would result. When harmol is added, about 1–2 μmol of this sulphate is used for conjugation, still leaving about 50% in the perfusion medium (Fig. 1). At the end of the perfusion virtually no harmol sulphate was found in the perfusion medium, indicating that at these low sulphate concentrations sulphation is no longer very efficient. At 2 h after its addition to the perfusion system [^{35}S]sulphate was still available for sulphation of harmol (see Fig. 1; control experiment).

At 0.65 and 1.30 mM-inorganic sulphate in the

perfusion medium 65 and 130 μmol of sulphate were presented to the perfused liver; presumably the concentration of sulphate in the liver would increase to the same value as that in the perfusion medium. At most 12 μmol of harmol sulphate was synthesized (Fig. 3b), and hence at most 12 μmol of inorganic sulphate was required; thus enough sulphate would be available. However, even at 0.65 mM-sulphate in the perfusion medium the sulphate concentration was not yet saturating, because at 1.30 mM-sulphate there was a 30–40% increase in the biliary excretion of harmol sulphate. This difference may be explained by a relatively high K_m for sulphate in the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate, the co-substrate of sulphation. The reported K_m values vary between 0.1 and 5 mM (Robbins, 1962; Hall & Straatsma, 1966; Levi & Wolf, 1969; Wong, 1976). If the K_m *in vivo* indeed is of the order of 0.4 mM (as suggested by our perfusion experiments) then our results can easily be explained by the K_m value for sulphate. Our rats have a serum inorganic sulphate concentration *in vivo* of 0.85 ± 0.04 mM. Exhaustion of sulphate certainly did not take place in the perfusion medium at the two higher sulphate concentrations.

Biliary excretion of inorganic sulphate and the harmol conjugates

At the higher concentrations of inorganic sulphate in the perfusion medium the concentration in bile equalled the concentration in the perfusion medium (Fig. 2), indicating that sulphate was 'passively' excreted in bile. The relatively high excretion at 0 mM-sulphate may have been due either to some form of 'active transport' or to adsorption of sulphate to some component that was actively excreted in bile.

Conclusion

Inorganic sulphate in the perfusion medium (and in plasma) mixes rapidly with the endogenous sul-

phate pool in the liver, resulting in about equal concentrations in both compartments. Conjugation with sulphate limits glucuronidation of harmol, especially at low harmol concentration. When inorganic sulphate concentration decreases, the rate of sulphation decreases, most likely because of a relatively high K_m of adenosine 3'-phosphate 5'-sulphatophosphate synthesis. Exhaustion of inorganic sulphate, though it may occur at extremely high doses of a substrate for sulphation, seems to play a less-important role.

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