The Availability of Inorganic Sulphate in Blood for Sulphate Conjugation of Drugs in Rat Liver *in vivo*

[³⁵S]SULPHATE INCORPORATION INTO HARMOL SULPHATE

By GERARD J. MULDER and EGBERT SCHOLTENS

Department of Pharmacology, State University of Groningen, Bloemsingel 1,
Groningen, The Netherlands

(Received 26 August 1977)

1. When Na₂³⁵SO₄ is injected intravenously in rats, it is immediately available for sulphate conjugation of the phenolic drug harmol (7-hydroxyl-1-methyl-9H-pyrido[3,4-*b*]indole) in the liver. This was established by following the time course of the biliary excretion of the sulphate conjugate of harmol, and the incorporation of [³⁵S]sulphate into harmol sulphate. 2. During the 10 min immediately after injection of Na₂³⁵SO₄ re-distribution of [³⁵S]sulphate took place, which resulted in a rapid initial decrease in the plasma concentration of [³⁵S]sulphate; a concomitant decrease in the amount of [³⁵S]sulphate incorporated into harmol sulphate was observed, indicating that the co-substrate of sulphonation, adenosine 3'-phosphate 5'-sulphatophosphate, equilibrates rapidly with [³⁵S]sulphate in plasma. 3. The results suggest that the pool size of adenosine 3'-phosphate 5'-sulphatophosphate is very small; therefore the specific radioactivity of [³⁵S]sulphate in plasma determines the specific radioactivity incorporated into sulphate esters at any time.

In mammals many drugs and other xenobiotics are metabolized to sulphate conjugates to various extents (Roy, 1971; Williams, 1974). The main site of sulphonation is the liver, but also other organs, such as kidney or brain, contain sulphotransferases (see Roy, 1971; Meek & Neff, 1973; Jansen *et al.*, 1973). Usually the sulphate conjugates are devoid of the pharmacological and toxicological properties of the parent compound, although the *N*-*O*-sulphate conjugates of *N*-hydroxy compounds form a notable exception to this: they are supposed to be important carcinogenic metabolites of hydroxamic acids and hydroxylamines (Weisburger & Weisburger, 1973; Kadlubar *et al.*, 1976). In most cases, however, the sulphate conjugates are rapidly eliminated from the mammalian body in urine or bile (Millburn, 1970).

The co-substrate of sulphonation, adenosine 3'-phosphate 5'-sulphatophosphate, is synthesized from ATP and inorganic sulphate in the liver and some other organs (see Roy, 1971). The steady-state concentration of adenosine 3'-phosphate 5'-sulphatophosphate in animal tissues is unknown, mainly due, it seems, to the instability of this compound and the lack of a sensitive assay. Such an assay, however, has been reported (Stanley *et al.*, 1975).

Similarly, no data are available on the rate of incorporation of inorganic sulphate through adenosine 3'-phosphate 5'-sulphatophosphate into drugs with an appropriate acceptor group for sulpha-

tion. Therefore we have investigated the rate of incorporation of ³⁵S-labelled inorganic sulphate into the harmol metabolite harmol sulphate. Harmol (7-hydroxy-1-methyl-9H-pyrido[3,4-*b*]indole) contains a phenolic hydroxyl group that can be conjugated with glucuronic acid or sulphate (Mulder & Hagedoorn, 1974).

The results show that inorganic sulphate in blood is immediately available for sulphonation of harmol, which takes place in the liver (Mulder *et al.*, 1975); the data suggest that the pool size of adenosine 3'-phosphate 5'-sulphatophosphate in liver is very small.

Materials and Methods

Rats

Male Wistar rats (195–205 g body wt.) were used throughout this study. They had free access to food and water. For the experiments they were anaesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally); artificial respiration was applied through a trachea cannula and a bile-duct cannula was inserted. The kidneys were ligated to prevent urinary excretion of harmol sulphate (Mulder *et al.*, 1975) and Na₂³⁵SO₄, which would complicate the analysis of the data. Bile was collected in fractions of 15 min. When plasma concentrations of ³⁵S-labelled sulphate were to be determined the carotid artery was can-

nulated and blood was collected in heparinized micro-tubes (Caraway Micro Blood Collecting Tubes, Sherwood Medical Industries, St. Louis, MO, USA). Plasma was obtained by centrifugation of the tubes (20 min at 1300g_{av.}). Injections of harmol or Na₂³⁵SO₄ were in the femoral veins; the compounds were dissolved in aq. 0.9% (w/v) NaCl and the rats received 2.5 ml of the solvent/kg body wt. intravenously. The dose of harmol was 20 µmol/kg, and that of Na₂³⁵SO₄ 50 µCi/kg. The amount of carrier sulphate injected was about 0.1 µmol/kg.

Materials

Harmol and other chemicals were from sources as given before (Mulder & Hagedoorn, 1974). Na₂³⁵SO₄ was from New England Nuclear, Dreieichenhain, West Germany. Of the stock solution in water (710 mCi/mmol initially), 10 µl was added to 5 ml of aq. 0.9% (w/v) NaCl, resulting in a 20 µCi/ml solution.

Separation and determination of harmol, harmol sulphate and inorganic sulphate

Harmol and its sulphate conjugate were separated as described by Mulder & Hagedoorn (1974) by applying a sample of bile or urine to a silica-gel t.l.c. plate (Merck AG, Darmstadt, West Germany; Silica Gel 60F 254), and chromatography with methanol/aq. NH₃ (sp.gr. 0.91)/chloroform/propan-2-ol (5:2.5:45:47.5, v/v). The R_F of harmol sulphate is 0.35–0.45 in this system. Inorganic sulphate remained completely at the site of application with this procedure, as tested by applying a sample of Na₂³⁵SO₄ to the plate. Harmol and its sulphate conjugate were determined fluorimetrically as described previously (Mulder & Hagedoorn, 1974). For determination of ³⁵S radioactivity a sample containing [³⁵S]sulphate was added to a scintillation medium (Plasmasol, New England Nuclear) and counted for radioactivity in a liquid-scintillation spectrometer. To determine ³⁵S radioactivity on the t.l.c. plate, spots were scraped off the plate after chromatography, and the scrapings were added directly to the scintillation medium for radioactivity counting; the presence of the silica-gel scrapings decreased the counting efficiency by about 15%. In some experiments 10–25 µl of plasma or bile was counted for radioactivity directly after addition of scintillation fluid without further work-up of the samples.

Results

Disappearance of [³⁵S]sulphate from plasma

After intravenous injection of Na₂³⁵SO₄ in rats with ligated kidneys an initial rapid disappearance of [³⁵S]sulphate from plasma was observed, indicating a rapid re-distribution of labelled sulphate from the blood. After about 10 min this initial phase was

Fig. 1. Disappearance of [³⁵S]sulphate from plasma in the rat

Na₂³⁵SO₄ (50 µCi/kg) was injected intravenously in a rat with ligated kidneys. Blood samples were taken from the carotid artery; the first sample was taken 60 s after the injection. A 25 µl sample of plasma was used for the determination of radioactivity. This is a typical example of the disappearance of [³⁵S]sulphate from plasma as determined in several rats.

complete and a steady-state of [³⁵S]sulphate was established (Fig. 1). It was slowly eliminated in bile (and, when the kidneys were left intact, in urine). In 2 h about 3% of the radioactivity had been excreted in bile, in rats with either intact or ligated kidneys. After 2 h about 8% of the radioactivity was found in urine in rats with intact kidneys. When the kidneys were left intact the rate of disappearance of [³⁵S]sulphate from the blood during the steady-state phase was only fractionally higher than in the rats with ligated kidneys.

Incorporation of [³⁵S]sulphate into harmol sulphate by sulphation

After administration of harmol to the rat two metabolites appear: the glucuronide and sulphate conjugates (Mulder & Hagedoorn, 1974). Both are excreted in bile and urine; when the kidneys are ligated the conjugates are completely eliminated in bile (Mulder *et al.*, 1975; Mulder & Scholtens, 1977). Fig. 2(a) shows the time course of biliary excretion of harmol sulphate in rats with ligated kidneys. During the four 15 min periods measured the excretion rate decreased slowly, in agreement with our previous results.

When Na₂³⁵SO₄ was given intravenously without harmol, considerable radioactivity was found in bile. After t.l.c. of a bile sample a small amount of radioactivity was found at the spot on the t.l.c. plate where harmol sulphate would have been if it had been present in the bile sample; most likely this represents a sulphate conjugate of some endogenous substrate of sulphation. Most of the radioactivity was found at

Fig. 2. Biliary excretion of harmol sulphate (a) and incorporation of [^{35}S]sulphate into harmol sulphate (b)

In (a) the biliary excretion of harmol sulphate after an intravenous injection of harmol (20 $\mu\text{mol/kg}$) is shown in rats with ligated kidneys; four 15-min fractions of bile were collected. Results are means $\pm$ S.E.M. (indicated by bars) for eight rats. The amount is expressed as percentage of the dose in that fraction (1% is 40 nmol of harmol sulphate). In (b) the radioactivity incorporated into harmol sulphate is given. A 10 μl sample of bile was subjected to t.l.c. and the harmol sulphate spot was scraped off the plate and counted for radioactivity. The total amount of radioactivity present at the harmol sulphate spot per 15-min fraction of bile is given for bile from rats that received: (A) only $\text{Na}_2^{35}\text{SO}_4$ (controls; means of three rats); (B) harmol and $\text{Na}_2^{35}\text{SO}_4$ simultaneously (four rats); and (C) harmol 30 min after $\text{Na}_2^{35}\text{SO}_4$ (three rats). $\text{Na}_2^{35}\text{SO}_4$ was given at zero time.

the site of application of the bile sample on the plate, where, indeed, inorganic sulphate remains: a 10 μl sample of bile gave a typical distribution of about 2000–4000 c.p.m. at the application site, and about 200–300 c.p.m. at the harmol sulphate spot when bile was used, obtained during the first 30 min after intravenous injection of 50 μCi of $\text{Na}_2^{35}\text{SO}_4/\text{kg}$. The amount of radioactivity at the site of application decreased slowly during the 90 min after the injection of $\text{Na}_2^{35}\text{SO}_4$ by 5–10%. The presence of harmol did not affect the amount of radioactivity found at the application site after t.l.c. Fig. 2(b) shows the radioactivity (after chromatography) at the harmol sulphate spot on the t.l.c. plate after application of a sample of bile from rats that had received: (A) only $\text{Na}_2^{35}\text{SO}_4$ and no harmol; (B) $\text{Na}_2^{35}\text{SO}_4$ and harmol simultaneously; (C) harmol 30 min after the injection of $\text{Na}_2^{35}\text{SO}_4$. Clearly, [^{35}S]sulphate was incorporated into harmol sulphate, and about twice the amount of radioactivity was incorporated into harmol sulphate in the rats that received harmol and $\text{Na}_2^{35}\text{SO}_4$ simultaneously, as compared with the rats that received harmol 30 min after $\text{Na}_2^{35}\text{SO}_4$. The amount of [^{35}S]sulphate incorporated into harmol sulphate decreased only slowly when harmol was injected 30 min after the injection of $\text{Na}_2^{35}\text{SO}_4$, in parallel with the decrease in the amount of harmol sulphate itself (Fig. 2a); however, when harmol and $\text{Na}_2^{35}\text{SO}_4$ were injected simultaneously, after the first 15 min period a pronounced fall in radioactivity excreted with harmol sulphate was observed.

To find out how long it took before [^{35}S]sulphate would be incorporated into harmol sulphate after injection of $\text{Na}_2^{35}\text{SO}_4$, we collected bile in 1-min fractions immediately after the simultaneous injection of harmol and $\text{Na}_2^{35}\text{SO}_4$ (Fig. 3). The rate of appearance of radioactivity in harmol sulphate was similar to the rate of appearance of harmol sulphate itself in bile; in all the individual experiments of Fig. 3, both rates reached a peak 4–6 min after the injection of harmol and [^{35}S]sulphate, and decreased slowly thereafter. The amount of radioactivity at the application site on the t.l.c. plate reached its peak later than that for harmol sulphate and remained constant thereafter. If no harmol was given only a small amount of radioactivity was found at the harmol sulphate spot (Fig. 3). Since the time between secretion of bile by the hepatocytes and its collection outside the body ('transit time') is about 1–2 min (Häcki & Paumgartner, 1973), these data indicate that almost immediately after the injection of $\text{Na}_2^{35}\text{SO}_4$ the radioactive sulphate is used for sulphonation of harmol.

Discussion

When $\text{Na}_2^{35}\text{SO}_4$ is given intravenously to rats it is rapidly incorporated into various endogenous tissue

Fig. 3. Rate of appearance of [^{35}S]sulphate in harmol sulphate excreted in bile after administration of $\text{Na}_2^{35}\text{SO}_4$. At zero time $\text{Na}_2^{35}\text{SO}_4$ was injected intravenously, either alone (a) or simultaneously with harmol (b). Bile was collected in 1-min fractions during 15 min. A $5\mu\text{l}$ sample of bile was applied to the t.l.c. plate, and after chromatography the amount of radioactivity at the site of application of the bile sample (●) and at the harmol sulphate spot (○) were counted for radioactivity. The amount of radioactivity in the spots derived from $5\mu\text{l}$ of bile is given. Comparison between (a) and (b) shows the amount of [^{35}S]sulphate incorporated into harmol sulphate. In (c) the time course of the biliary excretion of harmol sulphate itself (■) is given as the concentration of harmol sulphate in bile. The rats had ligated kidneys. Results in (a) are the means of two rats; those in (b) and (c) are means $\pm$ S.E.M. with five rats in each group.

components such as heparin, chondroitin sulphate (Stau *et al.*, 1973; Wood *et al.*, 1973), cartilage (Herbai, 1970) and the Golgi system (Katona, 1976). Less data are available on the incorporation of [^{35}S]sulphate into exogenously administered compounds. Hearse *et al.* (1969) have shown by radioautography that [^{35}S]sulphate was incorporated into aryl sulphate esters. Herbai (1970) has given details of [^{35}S]sulphate incorporation into phenyl sulphate in the mouse after intravenous administration of phenol. One of his conclusions was that administered [^{35}S]sulphate equilibrates rapidly, i.e. within 15 min, with the endogenous sulphate pool. The present work confirms this in the sense that after 10 min a steady state of [^{35}S]sulphate in plasma was established, during which sulphate was slowly eliminated, e.g. in urine and bile.

Before inorganic sulphate can be incorporated into harmol sulphate it must be incorporated into the co-substrate of sulphation, adenosine 3'-phosphate 5'-sulphatophosphate. Since our results show that [^{35}S]sulphate is immediately available for sulphate conjugation of harmol (Fig. 3), the pool of adenosine 3'-phosphate 5'-sulphatophosphate must be in rapid equilibrium with sulphate in plasma. Therefore the liver, in which harmol is sulphated, can draw directly from the pool of inorganic sulphate in plasma for sulphate conjugation of this type of xenobiotic. The plasma concentration of inorganic sulphate is about

0.5 mM. During the initial high plasma concentration of [^{35}S]sulphate, more labelling took place than later. If labelling of some substrate to be sulphated with a high specific radioactivity of [^{35}S]sulphate is required, it is therefore advisable to inject $\text{Na}_2^{35}\text{SO}_4$ and this substrate intravenously at the same time.

The following findings suggest that the pool size of adenosine 3'-phosphate 5'-sulphatophosphate is very limited or equilibrates extremely rapidly with sulphate in plasma. (1) During the 30 min after injection of $\text{Na}_2^{35}\text{SO}_4$ the initial high labelling of adenosine 3'-phosphate 5'-sulphatophosphate, observed when harmol and $\text{Na}_2^{35}\text{SO}_4$ were injected simultaneously (Fig. 2), has decreased again, presumably in parallel with the plasma concentration of [^{35}S]sulphate. Therefore adenosine 3'-phosphate 5'-sulphatophosphate cannot preserve a higher specific radioactivity in its sulphate group when the specific radioactivity of inorganic sulphate in plasma decreases. (Alternatively adenosine 3'-phosphate 5'-sulphatophosphate may only show a rapid turnover when there is a high demand for its sulphate group, e.g. induced by the presence of harmol; it will then be labelled with the specific radioactivity of [^{35}S]sulphate in plasma.) (2) The peak of radioactivity in harmol sulphate is reached at the same time as harmol sulphate itself reached its peak excretion rate (Fig. 3). If it took a minimum of several minutes to label the adenosine 3'-phosphate 5'-sulphatophosphate pool,

we would have found a slower rise in radioactivity in harmol sulphate, reaching a peak at a later time. (3) Although the excretion rate in bile of harmol sulphate decreases only slowly (Fig. 2), the radioactivity in harmol sulphate decreased rapidly when harmol and [³⁵S]sulphate were injected at the same time; when harmol was given after 30 min the decrease in radioactivity in harmol sulphate corresponded to the decrease in the amount of harmol sulphate itself. This suggests that during (part of) the initial period of 15 min after simultaneous administration of harmol and [³⁵S]sulphate, harmol sulphate is synthesized from adenosine 3'-phosphate 5'-sulphatophosphate with a higher [³⁵S]sulphate labelling (owing to a higher [³⁵S]sulphate concentration in plasma) than later. We have previously shown that 15 min after harmol injection sulphation of harmol still takes place (Mulder & Scholtens, 1977). At 10 min after its intravenous injection a steady state of [³⁵S]sulphate has been established, and harmol sulphate synthesized after that time is labelled with a constant radioactivity in its sulphate group.

These results cannot discriminate between a very small pool size of adenosine 3'-phosphate 5'-sulphatophosphate on the one hand, and an extremely rapid exchange of sulphate between plasma inorganic sulphate and adenosine 3'-phosphate 5'-sulphatophosphate on the other. However, the fact that two molecules of ATP are required for its synthesis would make a rapid synthesis and breakdown cycle of this co-substrate very unfavourable in terms of energy expenditure. Therefore, we tend to believe that the pool size is very small, rather than that its breakdown is very high. Moreover, because it is rather unstable

(Roy, 1971), a limited pool size might be an advantage for the organism, which, in that case, would synthesize adenosine 3'-phosphate 5'-sulphatophosphate only when it is required.

References

- Häcki, W. & Paumgartner, G. (1973) *Experientia* **29**, 1091–1093
- Hearse, D. J., Powell, G. M., Olavesen, A. H. & Dodgson, K. S. (1969) *Biochem. Pharmacol.* **18**, 205–209
- Herbai, G. (1970) *Acta Physiol. Scand.* **80**, 470–491
- Jansen, G. S. I. M., Van Elk, R. & Van Kempen, G. M. J. (1973) *J. Neurochem.* **20**, 9–12
- Kadlubar, F. F., Miller, J. A. & Miller, E. C. (1976) *Cancer Res.* **36**, 2350–2359
- Katona, E. (1976) *Eur. J. Biochem.* **63**, 583–590
- Meek, J. L. & Neff, N. H. (1973) *J. Neurochem.* **21**, 1–9
- Millburn, P. (1970) in *Metabolic Conjugation and Metabolic Hydrolysis* (Fisherman, W. H., ed.), vol. 2, pp. 1–74, Academic Press, New York
- Mulder, G. J. & Hagedoorn, A. H. (1974) *Biochem. Pharmacol.* **23**, 2101–2109
- Mulder, G. J. & Scholtens, E. (1977) *Biochem. J.* **165**, 553–559
- Mulder, G. J., Hayen-Keulemans, K. & Sluiter, N. (1975) *Biochem. Pharmacol.* **24**, 103–107
- Roy, A. B. (1971) *Handb. Exp. Pharmacol.* **28**, 536–563
- Stanley, P. E., Kelley, B. C., Tuovinen, O. H. & Nicholas, D. J. D. (1975) *Anal. Biochem.* **67**, 540–551
- Stau, T., Metz, J. & Taugner, R. (1973) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **280**, 93–102
- Weisburger, J. H. & Weisburger, E. K. (1973) *Pharmacol. Revs* **25**, 1–66
- Williams, R. T. (1974) *Biochem. Soc. Trans.* **2**, 359–377
- Wood, K. M., Wusteman, S. F. & Curtis, C. G. (1973) *Biochem. J.* **134**, 1009–1013