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THE DEPENDENCE OF THE RATE OF SULPHATE CONJUGATION ON THE PLASMA CONCENTRATION OF INORGANIC SULPHATE IN THE RAT *IN VIVO*

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Abstract—The dependence of the rate of sulphation of harmol [7-hydroxy-1-methyl-9*H*-pyrido(3,4-b)indole] on the plasma concn of inorganic sulphate was estimated in the rat *in vivo*. This was done in rats that were fed a low protein diet for 4 days to deplete the pool of inorganic sulphate, which decreased the plasma concn of sulphate from 0.9 mM normally to about 0.15 mM. Infusion of sodium sulphate at a rate that was stepwise increased, yielded a range of constant plasma sulphate levels below and above the physiological concn in plasma. By a constant infusion of harmol (6 μmoles/min/kg body wt), a steady state in the excretion of harmol sulphate could be reached at various plasma concns of sulphate. The apparent K_m for inorganic sulphate was approx 0.3 mM. The relevance of these data in the evaluation of the effects of changes in the availability of inorganic sulphate on sulphate conjugation of xenobiotics is discussed.

In mammals conjugation with inorganic sulphate is an important metabolic pathway in the elimination of various drugs and endogenous substances; in addition, sulphate conjugation is essential in the synthesis of some (macromolecular) body constituents like sulpholipids and glycosaminoglycans. The inorganic sulphate needed for sulphation has to be activated to the co-substrate of sulphate conjugation, adenosine 3′-phosphate 5′-sulphatophosphate (PAPS), before it can be transferred to the substrate. Sulphate is supplied to the body with food, as inorganic sulphate or through cysteine (and methionine), from which inorganic sulphate can be generated by sulphoxidation.

Under certain conditions the availability of inorganic sulphate will limit sulphate conjugation. Thus, exhaustion of the pool of sulphate occurs when food is deficient in sulphate and sulphur-containing amino acids [1]. It was also suggested that depletion of sulphate occurs when high doses of a drug that becomes sulphated are administered [2–4]. This suggestion came from the observation that an additional (oral or intravenous) supply of sulphate or a sulphate precursor increased sulphate conjugation of a drug *in vivo*. Indeed, upon administration of a high dose of drugs that are substrates for sulphation, the concn of inorganic sulphate in the blood was decreased considerably [1, 5–8]. In the present work we have tried to obtain information on the relationship between the sulphate concn in blood (plasma) in the rat *in vivo*, and the rate at which the phenolic compound, harmol, is sulphated during steady state infusion; harmol is completely metabolized in the rat via two competing metabolic pathways, conjugation with glucuronic acid or sulphate [9, 10]. When the apparent K_m for sulphate in the overall sulphation process is high, for instance equal to the physiological concn of sulphate in the blood, an additional supply of sulphate may raise the concn of sulphate above the physiological level and, thereby, increase the rate of sulphation. On the other hand, when the apparent K_m for sulphate is low relative to the physiological concn of sulphate in the blood, a decrease in the concn of sulphate will have little effect on the rate of sulphation up to a certain concn. The capacity of sulphate conjugation, however, may be limited because less sulphate is available; only when depletion of sulphate lowers the concn of sulphate below the threshold will replenishment of the pool of sulphate result in an increase in sulphation rate.

No *in vivo* data on the dependence of the rate of sulphation on plasma sulphate have been reported so far. In various *in vitro* preparations of rat liver (the isolated perfused liver, hepatocytes) very different apparent K_m values for sulphate were found, ranging from 0.15 to 3 mM [7, 11–13]. In the present study data are reported on the dependence of the rate of sulphation of harmol on the concn of sulphate in plasma in the rat in vivo.

MATERIALS AND METHODS

Materials. Harmol-hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). Bovine serum albumin (demineralized) was obtained from Poviet Producten (Oss, The Netherlands). All chemicals used were of analytical grade. The low-protein diet was obtained from Hope Farms (Woerden, The Netherlands). This diet consisted of: casein (84% protein), 8.0%; glucose (cerelose), 66.6%; corn starch (gelatinized), 10.0%; alpha cellulose, 5.0%; sunflower oil, 5.0%; calcium diphosphate, 2.5%; NaCl, 0.3%; KCl, 1.0%; MgO, 0.2%; choline chloride (50%), 0.4%; standard vitamin and trace element

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mixture (without inorganic sulphate), 1.0%. This diet, obtained as meal, was squeezed into lumps after addition of 20% tap water (w/w); the lumps were dried overnight at room temp., which caused a 50% loss of the water added.

Animals. Male Wistar rats of about 300 g body wt were used. They were fed a low-protein diet (see Materials) for 4 days prior to the experiment to deplete the pool of sulphate [1]. Rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). The temp. of the rats was kept between 37.5 and 38.5° by a heating pad. Artificial respiration was performed through a trachea cannula. The urine bladder was catheterized for continuous collection of urine. Blood was sampled through a catheter in the right carotid artery into heparinized microtubes (Caraway Micro Blood Collecting Tubes, Sherwood Medical, St. Louis, MO, U.S.A.). The experiment was started with a single intravenous injection of harmol (133 μmoles/kg). Thereafter, a constant infusion of harmol (approx. 6 μmoles/min/kg body wt) in aq. 0.9% (w/v) NaCl was given (about 0.53 ml/min/kg) through a catheter in the left jugular vein. The infused solution also contained bovine serum albumin (0.3%, w/v), to compensate for the loss in blood proteins by blood sampling, and D-mannitol (about 220 μmoles/min/kg) to stimulate urine production.

From prior experiments [14] it was known that infusion of comparable amounts of harmol resulted in complete cholestasis after approx. 2 hr; this caused an abrupt decrease in the excretion of harmol glucuronide that is predominantly excreted in bile. It had only little effect on the excretion of harmol sulphate that is largely excreted in urine. Later on the elimination of harmol glucuronide by excretion in urine compensated for the loss of biliary excretion. To avoid this shift in excretory pathway during the experiment, which made steady-state studies impossible, the bile duct was ligated from the beginning of the experiment so that a steady-state excretion of harmol glucuronide in urine was established.

To achieve different plasma concns of inorganic sulphate in the rat, sodium sulphate was added to the infused solution in a concn that was increased stepwise (from 0.0 to 7.6 mM sodium sulphate), resulting in a sulphate infusion rate of 0.0–4.0 μmoles/min/kg; every sulphate infusion period (45 min) was started with a single intravenous injection of sodium sulphate that increased proportionally to the rate of infusion of sulphate (30–240 μmoles/kg), to obtain rapidly a new steady-state in the plasma concn of sulphate (see also Fig. 1). At the end of every infusion period, and 15 min before, blood was sampled to obtain plasma for the determination of the plasma concn of sulphate. In addition, at the end of every period an additional blood sample was taken to determine the concn of unconjugated harmol in whole blood. Determination of the concn of free harmol was performed on the day of the experiment. Plasma and urine samples were stored at −30°.

Analytical procedures. The conjugates of harmol were quantitated fluorometrically after thin layer chromatography by the method of Mulder and Hagedoorn [15]. The concn of free harmol in whole blood was determined as follows: after addition of 100 μl 0.2 M sodium phosphate buffer (pH 9.5) to 200 μl of whole blood, free harmol was extracted with 3 ml ethyl acetate; the harmol in 2 ml of the ethyl acetate layer was extracted into 3 ml 0.1 N HCl and measured fluorometrically as described by Mulder and Hagedoorn [15].

For determination of the concn of inorganic sulphate, plasma samples were deproteinized by elution over a Bio-Gel P-2 (100–200 mesh) column with 5 mM aq. $(NH_4)HCO_3$ as eluent, and the inorganic salt fractions was collected. Anions in this fraction were separated on a column containing low capacity anion-exchange resin (developed by Dr. B. P. Knol, Department of Analytical Chemistry, State University of Groningen, The Netherlands), in series with an anion suppressor column (Dowex 50W–X8; 200–400 mesh). The eluent contained 2.5 mM Na_2CO_3 in water and the flow rate was approx. 15 ml/hr. The sulphate ions were quantitated conductometrically. Recently a comparable microassay of inorganic sulphate in biological fluids by controlled flow anion chromatography was reported by Cole and Scriver [16].

RESULTS

To investigate the dependence of sulphation rate on the plasma concn of inorganic sulphate in the rat *in vivo*, a constant infusion of harmol (about 6.0 μmoles/min/kg body wt) was given at various sulphate levels in plasma. The only metabolites of harmol are its sulphate and glucuronide conjugates [9]. The rate of sulphation of harmol could be estimated from the amounts of harmol sulphate excreted in urine at steady state excretion of the sulphate conjugate; since the bile duct was ligated (to avoid complications of cholestasis by harmol glucuronide; see Materials and Methods) biliary excretion was not possible.

For these experiments we used rats that were depleted in sulphate by feeding a low-protein diet for the 4 days prior to the experiment, which does not affect sulphation and glucuronidation of harmol in the rat [14]. This resulted in a plasma concn of sulphate of 0.15–0.20 mM (while it was approx. 0.9 mM in normally fed rats). From that situation the sulphate level was increased stepwise (at 45 min intervals) by intravenous infusion of sodium sulphate. In this way a range of plasma concns of sulphate was obtained from 0.1 to 2.4 mM. During the infusion of harmol, the concn of unconjugated harmol in whole blood varied between 70 and 85 μM; it remained constant, or showed only a slow increase, during the experiments (10–20% increase in 5 hr). During the last 15 min of every infusion period, before the infusion rate of sodium sulphate was increased, the plasma concn of sulphate was constant and the urinary excretion of harmol sulphate was in steady state. The stepwise increase in the plasma concn of sulphate in the rat caused a concomitant stepwise increase in the excretion of harmol sulphate (a typical experiment is shown in Fig. 1). Simultaneously, the excretion of harmol glucuronide gradually decreased during the experiment.

At the lowest plasma concn of sulphate (about

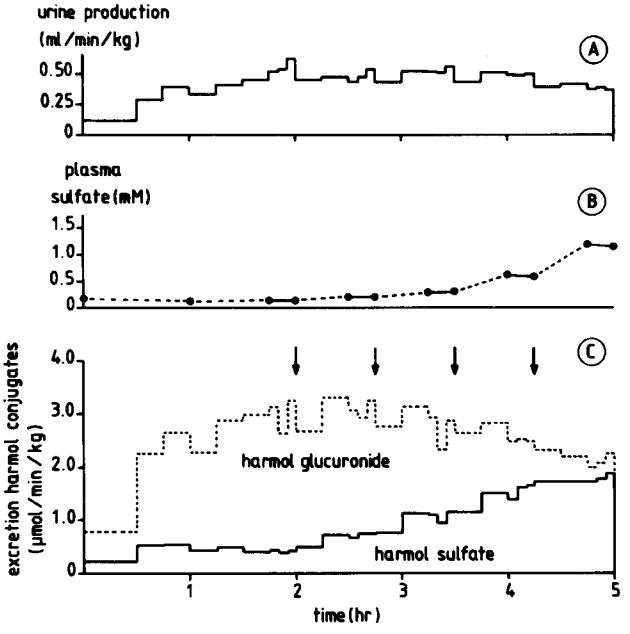

Fig. 1. Urine production (a), plasma sulphate levels (b) and excretion of harmol conjugates in urine (c) at constant infusion of harmol (6 μmoles/min/kg body wt) in the rat *in vivo*. Results of a typical experiment are shown. Rats were fed a low-protein diet for the 4 days prior to the experiment to deplete sulphate. In the lowest panel (c) the excretion of harmol sulphate (——) and harmol glucuronide (- - -) in urine is indicated. At the arrows infusion of sodium sulphate was started in increasing amounts (0.5, 1.0, 2.0 and 3.0 μmoles/min/kg, respectively); every sulphate infusion period (45 min) was started with an intravenous injection of sodium sulphate (30, 60, 120 and 180 μmoles/kg, respectively). At 15 min before the end of each period, and at the end of every sulphate infusion period, blood was obtained to determine the concn of inorganic sulphate in plasma (panel b).

0.1 mM) only small amounts of harmol sulphate were excreted (0.3–0.4 μmoles/min/kg) (Fig. 2). A continuous and steep increase in the rate of sulphation of harmol was observed when the plasma concn of sulphate was increased to about 0.6 mM; the sulphation rate was increased 4–5-fold and amounted to 1.2–1.6 μmoles/min/kg. At sulphate levels above 0.6 mM a relatively small additional increase in the sulphation rate was measured. In rats fed a normal diet, infusion of huge amounts of sulphate (up to 55 μmoles/min/kg) caused little or no additional increase in the excretion of harmol sulphate (results not shown), although the plasma concn of sulphate was raised above 10 mM. From these data an estimation can be made of the apparent K_m for inorganic sulphate in the overall sulphation of harmol: approx. 0.3 mM. The maximal rate of sulphation of harmol at the infusion level of harmol used in these experiments amounted to about 2 μmoles/min/kg body wt.

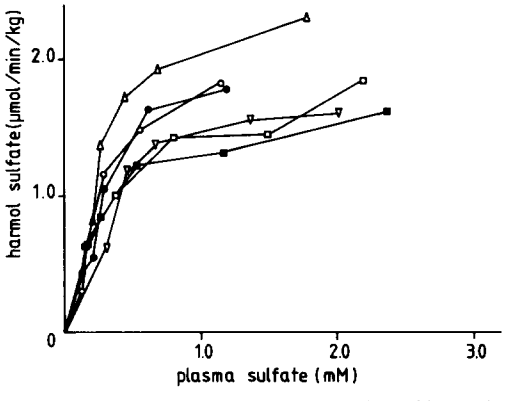

Fig. 2. Dependence of the rate of sulphation of harmol on the plasma concn of inorganic sulphate in the rat *in vivo*. Urinary excretion of harmol sulphate during steady-state metabolism of a constant infusion of harmol (i.v. 6 μmoles/min/kg body wt) in relation to the plasma concn of sulphate. Results are shown for six individual rats.

DISCUSSION

In the present study the dependency of the rate of harmol sulphation on the plasma concn of inorganic sulphate was investigated. Especially when plasma sulphate was below the physiological concn the rate of sulphation increased considerably at increasing concns of sulphate. At concns of sulphate above the physiological level the excretion of harmol sulphate levelled off; in these experiments the urinary excretion of harmol sulphate was not rate limiting. An apparent K_m for sulphate of the overall sulphation reaction (sulphate activation and sulphate transfer) for harmol was estimated to be 0.3 mM; in the literature no comparable *in vivo* data are available. Since harmol is thought to be predominantly conjugated in the liver, it is interesting that a similar apparent K_m for sulphate (approx. 0.4 mM) in the sulphation of harmol was found in the isolated perfused rat liver preparation [11].

The relation between the concn of sulphate and the rate of sulphation of xenobiotics has been inves-

tigated more extensively with isolated hepatocytes from the rat. However, with this preparation widely different values for the apparent K_m for sulphate were found, varying between 0.15 and 3 mM. Unfortunately, various substrates for sulphation were used and, therefore, the obtained apparent K_m values are difficult to compare: 3 mM with paracetamol [12], 0.47 mM with salicylamide [7] and about 0.15 mM with 1-napthol [13]. If the PAPS synthesis is the rate-limiting step in sulphation, then one would expect the K_m for sulphate to be the same for every substrate. However, multiplicity in phenol sulphotransferases [17, 18] may explain a different apparent K_m for sulphate for various substrates when the sulphotransferases involved differ in their affinity for PAPS.

The present results in the rat suggest that a decrease in the concn of sulphate in blood below the physiological level must affect the rate of sulphation of various phenolic compounds with an apparent K_m for inorganic sulphate similar to that of harmol. A deficiency of inorganic sulphate may also impair the synthesis of sulphated macromolecules, like glycosaminoglycans and sulpholipids and, thereby, cause retardation of growth and development. On the other hand, it may have important implications for the detoxication and elimination of various drugs. In case of sulphate depletion by an overdose of a drug (for instance paracetamol), it may be beneficial to replenish the pool of sulphate and increase the plasma concn of sulphate to stimulate the elimination of a drug. A rapid increase of sulphation can be expected upon administration of sulphate (or a precursor) as shown in this study and recently by Galinsky and Levy [19]. However, raising the plasma concn of sulphate above the physiological level will have only a little effect on the sulphation rate of drugs like harmol. This might explain the limited effect of a high inorganic infusion on sulphation of phenol [20].

Extension of these findings to other phenolic compounds will no doubt increase the insight into the kinetics of sulphation and make the interpretation of earlier findings easier.

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