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D-CYSTEINE AS A SELECTIVE PRECURSOR FOR INORGANIC SULFATE IN THE RAT *IN VIVO*

EFFECT OF D-CYSTEINE ON THE SULFATION OF HARMOL

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Abstract—D-Cysteine, the unphysiological isomer of the sulfur containing amino acid L-cysteine, is not utilized for protein synthesis, glutathione synthesis or taurine production; it was tested as a selective precursor for inorganic sulfate, required for sulfation of xenobiotics. Both cysteine isomers were injected intravenously in the rat, in order to investigate their sulfoxidation to inorganic sulfate. The rates of sulfoxidation were very similar, so that stereospecificity for the amino acid seemed not to play a role. When the rats were fed a low-protein diet (LP-diet; containing only 8% casein as source of amino acids) the serum sulfate concentration decreased to about 20% of control. Under these circumstances the rate of sulfoxidation of both isomers was decreased to the same extent. In order to confirm that both cysteine isomers were equally efficient in providing inorganic sulfate for sulfation of xenobiotics, a constant infusion of harmol (a substrate for sulfation) was given to rats fed the LP-diet. Administration of L- or D-cysteine yielded similar increases in sulfation of harmol under these conditions. These results show that D-cysteine can be used to selectively enhance sulfate availability.

Sulfation is an important route of metabolism of many xenobiotics [1]. It requires inorganic sulfate which, under normal conditions in mammals, is chiefly derived from the oxidation of L-cysteine. The metabolism of L-cysteine *in vivo* has been well studied [2–4]. It is incorporated into protein and peptides like glutathione, and converted by various pathways to several metabolites, one of which is inorganic sulfate. This inorganic sulfate is essential for sulfate conjugation of not only xenobiotics but also endogenous substances, such as glycosaminoglycans of connective and bone tissues [5].

Because L-cysteine is utilized for the synthesis of cosubstrates for several conjugations (taurine, glutathione and sulfate conjugation), we wanted to investigate the use of its unphysiological isomer D-cysteine as a selective precursor for inorganic sulfate, since D-cysteine is not converted to taurine or glutathione or utilized for protein synthesis.

We have previously shown that both L- and D-cysteine are converted to inorganic sulfate when these isomers are administered orally in the rat [6]. Interestingly, D-cysteine yielded a high serum sulfate level much earlier than L-cysteine, presumably because the concentration of D-cysteine in serum was much higher than that of L-cysteine after oral administration. It is difficult to determine the cause of the difference in behaviour between the isomers, since the rate of uptake from the gut, first pass metabolism in the liver, or the rate of sulfoxidation might be different. Indeed, Ewetz and Sorbö [7] concluded from *in vitro* studies that sulfoxidation was highly stereospecific, the rate for the D-isomer being only 10% of that for the L-isomer.

In the present work we have administered both isomers intravenously in order to prevent complications at the level of intestinal drug absorption. We have determined the rate of sulfoxidation of both isomers by measuring the rate of sulfate accumulation in blood in rats with ligated kidneys.

Furthermore, we have studied the sulfate conjugation of harmol after an intravenous injection of both isomers of cysteine, under circumstances in which little sulfate is available.

MATERIALS AND METHODS

Chemicals. L-Cysteine–HCl, D-cysteine–HCl and harmol–HCl were obtained from Sigma Chemical Company (St. Louis, MO, USA). The normal pelleted rat food (RMH-B) and the low-protein diet (LP-diet) were obtained from Hope Farms (Woerden, the Netherlands). The composition and preparation of the LP-diet has been described by Krijgsheld *et al.* [8].

Rats. Male Wistar rats (approx. 250–300 g) were used throughout the experiments. They received a normal pelleted food or (when indicated) the LP-diet. The rats had free access to tap water. Before the experiments they were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The body temperature was kept between 37.5 and 38.5° by a heating pad. Artificial respiration was applied to the rats, and the carotid artery was catheterized to sample blood.

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Blood was collected in heparinized microtubes, approx. 0.3 ml per sample. The procedures have extensively been described elsewhere [9].

Unless otherwise stated, during the experiments in which the rate of sulfoxidation was determined, the kidneys were ligated to prevent urinary excretion of inorganic sulfate. These experiments were started by injection of L- or D-cysteine–HCl in a lateral tail vein at a dose of 1.5 mmoles/kg rat. These cysteine solutions were adjusted to pH 7 by a 2 M NaOH solution. Control rats received 1.5 mmol/kg NaCl.

For the experiments in which the sulfation of harmol was studied, bile duct and urine bladder were catheterized for continuous collection of bile and urine. Through a catheter in the left external jugular vein an infusion was given of D-mannitol (75 mg/ml) in aqueous 0.9% (w/v) NaCl at a rate of 9.5 ml/hr, to stimulate the urine production [9]. At $t = 0$ the experiment was started with an injection of 40 μmoles/kg harmol in the left external jugular vein, followed by an infusion of harmol (100 μmoles/hr/kg, dissolved in the D-mannitol infusion solution). At $t = 90$, L-cysteine, D-cysteine or NaCl (1.5 mmoles/kg) were administered as described in the previous paragraph. Bile and urine were collected in fractions of 30 min.

Assays. Inorganic sulfate in plasma that was deproteinized by Bio-Gel P-2 column chromatography, was estimated by conductometry after separation of the inorganic anions as described elsewhere [10]; the method is very similar to that described by Cole and Scriver [11].

The conjugates of harmol in bile and urine were separated by thin layer chromatography according to Mulder and Hagedoorn [12]. The concentration of these conjugates was determined fluorimetically in a Perkin–Elmer 1000 M Fluorescence Spectrophotometer, equipped with an interference filter that transmitted light at a wavelength of 374 nm ± 1% and a cut-off filter, that transmitted light above 390 nm.

RESULTS

Conversion of L- *and* D-*cysteine to inorganic sulfate.* To determine the rate at which cysteine is sulfoxidated *in vivo* to inorganic sulfate, L- and D-cysteine were injected intravenously in rats in which the kidneys were ligated. Under these circumstances the rate of the increase in the plasma sulfate level reflects the rate of sulfate synthesis. Figure 1 shows that both L- and D-cysteine increased plasma inorganic sulfate concentrations at very similar rates, although, after D-cysteine the initial rate of sulfate formation tends to be somewhat slower. Injection of an equimolar amount of NaCl did not have any influence upon plasma sulfate levels. Five hours after the injection of both isomers, plasma sulfate concentration had not yet reached its maximum. This result was confirmed by experiments in freely moving rats with ligated kidneys. In these rats plasma sulfate levels increased up till 4 mM, 12 hr after injection of each of the cysteine isomers (results not shown).

These experiments show that under these conditions little difference in sulfoxidation rates can be detected. In order to investigate the influence of

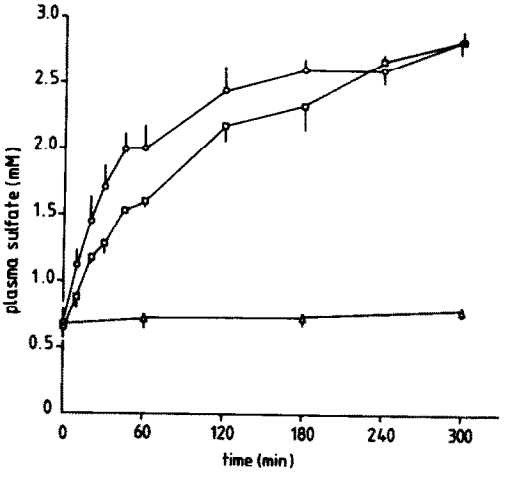

Fig. 1. Increase in plasma inorganic sulfate in rats with ligated kidneys after i.v. administration of L-cysteine (○—○), D-cysteine (□—□) or sodium chloride (△—△). The dose was 1.5 mmoles/kg: the rats were fed a normal rat food.

ligation of the kidneys, we repeated the experiments of Fig. 1 in rats with intact kidneys. Figure 2 shows that there still was no difference in the rate at which L-, or D-cysteine was converted to inorganic sulfate. However, it should be noted that in rats with intact kidneys the scatter of the results (judged by the SEM) is much greater, probably due to differences in the rates of urinary excretion of inorganic sulfate.

Effect of a low-protein diet on sulfoxidation of cysteine. When a diet is given that is low in sulfur-containing amino acids, the cysteine supply becomes rate limiting for protein synthesis, and it might be expected that as much cysteine as possible would be "saved" from sulfoxidation. We have repeated the experiment of Fig. 1 in rats that had been fed an 8% casein diet, which is deficient in cysteine and methionine. Although the rats did grow slowly on this diet, their serum sulfate level was much

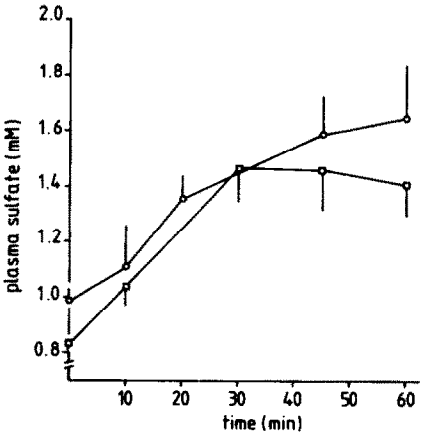

Fig. 2. The increase in plasma inorganic sulfate in rats with intact kidneys on a normal rat food. L-Cysteine (○—○) and D-cysteine (□—□) were injected i.v. at a dose of 1.5 mmoles/kg.

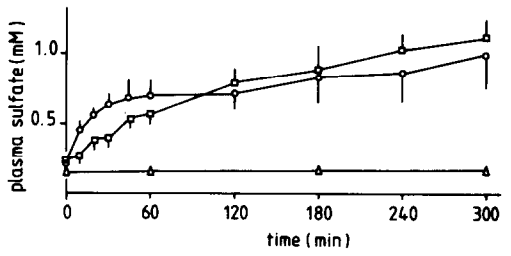

Fig. 3. The effect of a low-protein diet on sulfoxidation of L-cysteine (○—○) or D-cysteine (□—□). The controls received sodium chloride (△—△). The dose was 1.5 mmoles/kg i.v.. The rats had been on the diet for 4 days.

decreased [8]. In these rats the sulfoxidation of both L- and D-cysteine was slower than in normal rats, although after 5 hr there still was a slow increase in plasma sulfate (Fig. 3); no significant difference between the two isomers with regard to their sulfoxidation rates was detectable; again, the initial rate of sulfate formation from L-cysteine seems to be somewhat faster.

Effect of L- *and* D-*cysteine on sulfation of harmol in rats on a LP-diet.* When harmol is infused at a rate of 100 μmoles/hr/kg in rats fed a normal diet, more than 50% of the dose is excreted as the sulfate conjugate [13]. However, rats that are fed the LP-diet, excreted only about 10% of the same dose as the sulfate conjugate (Fig. 4). A steady state in the excretion of the harmol conjugates, harmol sulfate and harmol glucuronide, was reached 1 hr after the experiment was started. After 90 min, an intravenous injection of L- or D-cysteine was given; almost instantaneously the total amount of harmol sulfate in bile and urine increased, with a concomitant decrease in the excretion of harmol glucuronide. The total excretion remained at the same level. The amount of harmol sulfate formed increased considerably during the first 90 min after L- and D-cysteine injection (Fig. 4). During the last hour of the experiment the excretion of both harmol sulfate and harmol glucuronide was virtually constant; more that 50% of the amount of excreted conjugates consisted of harmol sulfate. Control rats that had received 1.5 mmoles/kg NaCl, excreted a constant fraction of 10% of the total amount of conjugates as the sulfate conjugate.

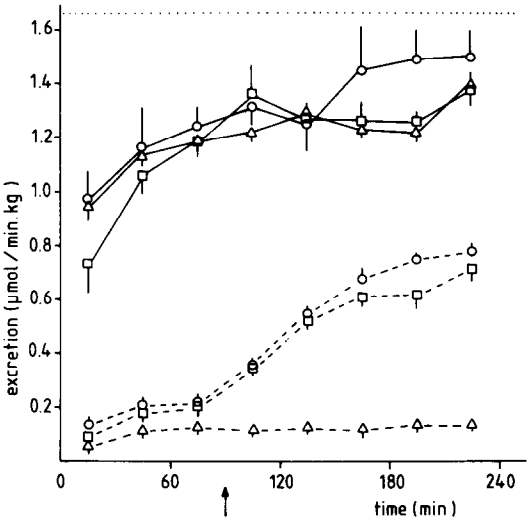

Fig. 4. The effect of a low-protein diet on sulfation of harmol. During the whole experiment an infusion of 1.67 μmoles harmol/min/kg was given (dotted line). The total excretion of harmol sulfate and harmol glucuronide in bile and urine in periods of half an hour was determined and expressed as mean excretion per minute (unbroken lines), dashed lines give the excretion of harmol sulfate. At $t = 90$ min (see arrow) L-cysteine (○), D-cysteine (□) or NaCl (△) were injected intravenously at a dose of 1.5 mmoles/kg.

In rats that were fed the LP-diet, the plasma sulfate concentration had decreased to 0.2–0.3 mM, as compared with about 1.0 mM in normal rats [8]. Under these circumstances the plasma sulfate concentration changed little during the harmol infusion (Fig. 5). The intravenous injection of 1.5 mmoles/kg of L- or D-cysteine, as expected, rapidly increased the plasma sulfate concentration. NaCl had no effect on the low plasma sulfate concentration.

DISCUSSION

The D-isomer of the sulfur-containing amino acid cysteine seems interesting as a selective precursor for inorganic sulfate, because it cannot be converted to taurine or glutathione, and cannot be utilized for protein synthesis like the L-isomer of cysteine. Therefore under circumstances in which the sulfur-supply is low, sulfation can be selectively increased by D-cysteine as a sulfate precursor.

When the isomers are administered intravenously, we find little difference in the rates of sulfoxidation of L- and D-cysteine to inorganic sulfate. Thus, the stereospecificity reported, based on *in vitro* experiments [7], does not seem to be relevant in our *in vivo* system. One explanation may be that the isomers follow different pathways to inorganic sulfate, which just happen to have the same rate. However, when rats are fed a LP-diet the sulfoxidation of both L- and D-cysteine is decreased to approximately the same extent. This finding suggests that the same enzyme system is involved in the sulfoxidation of both isomers. In fact, it is somewhat surprising that equal amounts of L-cysteine and D-cysteine are converted to inorganic sulfate, because the high requirement for L-cysteine under the LP-diet conditions would be expected to lower the substrate availability of this isomer specifically, while there would be no such effect on D-cysteine.

Previously we have reported [6] that D-cysteine did not increase the taurine concentration in serum, while L-cysteine administration resulted in the expected increase. The first step in the metabolic pathway from L-cysteine to both inorganic sulfate and taurine is catalyzed by the same enzyme, namely cysteine dioxygenase, leading to the first intermediate, alanine-3-sulfinate [2]. From this intermediate different pathways lead to, ultimately, sulfate or taurine. Therefore, we conclude that the stereospecificity observed in the formation of taurine from L- and D-cysteine is most likely not caused by the enzyme cysteine dioxygenase, but probably by the enzymes which catalyze the decarboxylation of alanine-3-sulfinate to hypotaurine, or the formation

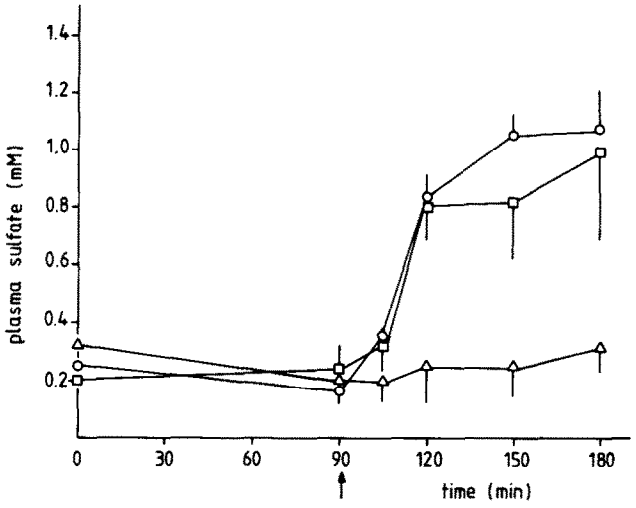

Fig. 5. The effect of an intravenous injection of L-cysteine (○), D-cysteine (□) or NaCl (△) on inorganic sulfate concentrations in plasma. The rats were the same as used in the experiment of Fig. 4.

of taurine from hypotaurine. Furthermore, the data suggest that there possibly is no stereospecificity in the transamination or the oxidative deamination of alanine-3-sulfinate, which is required for the formation to inorganic sulfate.

The very different levels of inorganic sulfate found previously early after oral administration of L- and D-cysteine [6] most likely are due to differences in the handling of the isomers before they reach the general circulation. In the present work equal amounts of both isomers reach the general circulation at the same time, so that no difference in the build-up of the concentration of the isomers in blood (as observed after oral administration) occurs. The differences between L- and D-cysteine observed previously, therefore, may be due to slower absorption of the L-isomer, or to a high first pass removal of L-cysteine during the first passage through gut mucosa and liver. In this respect a further study on the metabolism of L- and D-cysteine by the gastrointestinal tract seems of interest.

The inorganic sulfate formed from L- or D-cysteine is immediately available for sulfation of drugs, like harmol. When an infusion of harmol of 1.65 μmoles/min/kg is administered to LP-rats, approximately 0.20 μmoles/min/kg is excreted as harmol sulfate (Fig. 4); on a normal diet rats synthesize about 1.15 μmoles/min/kg harmol sulfate [13]. When L-cysteine is injected intravenously into LP-rats, the rate of formation of harmol sulfate increases to approximately 0.75 μmoles/hr/kg during steady state. As in the experiments without harmol (Fig. 3), plasma sulfate concentrations immediately increased after the injection of L-cysteine till ultimately a concentration of about 1 mM is reached, a value that is about 5 times as high as the original concentration in the LP-rats.

The results, obtained with D-cysteine were at any time comparable with those of L-cysteine (Figs. 4 and 5). This means that intravenously injected D-cysteine is capable to replace L-cysteine with equal efficiency as precursor of inorganic sulfate, and that it can support sulfate conjugation equally effectively. Thus, for studies in which highly selectively only sulfation is to be increased, while taurine and GSH conjugation should be unaffected [6], D-cysteine may be a suitable tool to make this possible.

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REFERENCES

1. G. J. Mulder, in *Sulfation of Drugs and Related Compounds* (Ed. G. J. Mulder), p. 1. CRC Press Inc., Boca Raton, FL (1981).
2. T. P. Singer, in *Metabolism of Sulfur Compounds* (Ed. D. M. Greenberg), 3rd edn., p. 535. Academic Press, New York, N. Y. (1975).
3. L. E. Benjamin and R. D. Steele, *Am. J. Physiol.* **241**, G503 (1981).
4. M. H. Stipanuk, *J. Nutr.* **109**, 2126 (1979).
5. R. H. de Meio, in *Metabolism of Sulfur Compounds* (Ed. D. M. Greenberg), 3rd edn., p. 287. Academic Press, New York (1975).
6. K. R. Krijgsheld, E. J. Glazenburg, E. Scholtens and G. J. Mulder, *Biochim. biophys. Acta* **677**, 7 (1981).
7. L. Ewetz and B. Sörbo, *Biochim. biophys. Acta* **128**, 296 (1966).
8. K. R. Krijgsheld, E. Scholtens and G. J. Mulder, *Biochem. Pharmac.* **30**, 1973 (1981).
9. G. J. Mulder, E. Scholtens and D. K. F. Meijer, in *Methods in Enzymology* (Ed. W. B. Jakoby), Vol. 77, p. 21. Academic Press, New York (1981).
10. K. R. Krijgsheld, E. Scholtens and G. J. Mulder, *Biochem. Pharmac.* **31**, 3997 (1982).
11. D. E. Cole and C. R. Scriver, *J. Chromat.* **225**, 359 (1981).
12. G. J. Mulder and A. H. Hagedoorn, *Biochem. Pharmac.* **23**, 2101 (1974).
13. H. Koster, I. Halsema, E. Scholtens, M. Knippers and G. J. Mulder, *Biochem. Pharmac.* **30**, 2569 (1981).