THE EFFECT OF CYSTEINE ANALOGUES ON THE EXCRETION OF URINARY SULPHATE IN THE RAT FOLLOWING CYSTEINE ADMINISTRATION

E.S. Roopnarinsingh[1], G.B. Steventon[2], R.M. Harris[3], R.H. Waring[3] and S.C. Mitchell[1]*

[1]*Section of Biological Chemistry, Division of Biomedical Sciences, Faculty of Medicine, Imperial College London,*
[2]*Department of Pharmacy, School of Health and Life Sciences, King's College London, London and* [3]*School of Biosciences, University of Birmingham, Birmingham, UK*

SUMMARY

A major pathway for the production of sulphate within the mammalian body is known to be via the oxidative degradation of the sulphur moiety within the amino acid, L-cysteine. The ability of two structurally similar sulphur-containing drugs, the anti-rheumatic agent, D-penicillamine, and the mucoactive compound, *S*-carboxymethyl-L-cysteine, to interfere with this sulphate production was investigated. Co-administration to the male rat of D-penicillamine (p.o.) and *S*-carboxymethyl-L-cysteine (p.o.) with [^{35}S]-L-cysteine (i.p.) led to a significant decrease in the subsequent urinary elimination of inorganic sulphate whilst having no measurable effect on organic sulphate excretion. The co-administration of L-valine, an amino acid not containing sulphur, had no effect. It is not known where, within the complex sequence of events surrounding the degradation of cysteine to

* Author for correspondence:
Dr. S.C. Mitchell
Section of Biological Chemistry
Division of Biomedical Sciences
Faculty of Medicine
Imperial College London,
Sir Alexander Fleming Building
South Kensington
London SW7 2AZ, UK
e-mail: s.c.mitchell@imperial.ac.uk

sulphate, that D-penicillamine or *S*-carboxymethyl-L-cysteine may interact.

KEY WORDS

sulphate, metabolism, L-cysteine, D-penicillamine, *S*-carboxymethyl-L-cysteine, rat

INTRODUCTION

Sulphate has been recognised for many years as a major sulphur-containing excretory product in animal urine /1/. Furthermore, its presence as both 'organic sulphate', bound through an ether-type linkage, and as 'free sulphate', the inorganic sulphate ion, has been widely appreciated /2/. The absorption of inorganic sulphate from the gastrointestinal tract is relatively inefficient and is an easily saturated process /3,4/; thus the preponderance of sulphate required for intermediary metabolism, and that which eventually appears in the urine, is produced mainly from sulphur-containing compounds within the body. The amino acid, cysteine (Fig. 1), is considered to be the chief provider, and pathways of intermediary metabolism have been proposed describing the stepwise exhaustive oxygenation of cysteine sulphur to sulphate /5-7/. It has also been shown that certain cysteine analogues may likewise be degraded to yield sulphate, presumably by identical or similar metabolic pathways /8/. The sulphur moieties of two such compounds, D-penicillamine, an anti-rheumatic drug, and *S*-carboxymethyl-L-cysteine, a mucoactive agent, are known to be degraded in part to yield sulphate /9-12/. However, it is not understood whether these compounds are able to interfere with the conversion of cysteine itself to sulphate. This aspect has now been investigated in the present study following their co-administration with [^{35}S]-L-cysteine to adult male rats.

TABLE 1

Urinary excretion of radioactivity from pre treated rats following [^{35}S]-L-cysteine administration

Pre-treatment	No. of animals	Recovery	Total sulphate	Organic sulphate	Inorganic sulphate
No treatment (control)	20	38.6 ± 8.8	37.3 ± 8.9	11.5 ± 4.3	25.8 ± 7.5
L-Valine	6	34.3 ± 14.2	33.9 ± 14.4	11.9 ± 3.2	22.0 ± 11.6
D-Penicillamine	6	28.3 ± 7.4*	27.6 ± 7.7*	10.7 ± 2.4	16.9 ± 6.6*
***S*-Carboxymethyl-L-cysteine**	8	29.8 ± 9.3*	28.4 ± 9.6*	9.1 ± 2.2	19.2 ± 8.5*

Values given as percentage radioactivity administered excreted in 0-24 h urine.
* Values were significantly different from controls (Student's t-test; 0.050>p>0.025).

MATERIALS AND METHODS

Chemicals

L-Cysteine hydrochloride, D-penicillamine (3-mercaptovaline), *S*-carboxymethyl-L-cysteine (carbocisteine; 3-[(carboxymethyl) thio]aniline), L-valine (2-aminoisovaleric acid), polyoxyethylene lauryl ether, zinc sulphate and barium chloride were supplied by Sigma-Aldrich Chemical Co. (Gillingham, Dorset). Corn oil was purchased from a local store. Other chemicals used were of analytical grade, with solvents being of h.p.l.c. quality, and all were readily available within the laboratory. Radiolabelled [^{35}S]-L-cysteine hydrochloride (sp. act. 103 mCi/mol, purity >99%) was obtained from Amersham International plc., Amersham, UK)

Animal investigations

Forty male Wistar rats (200 ± 12 g; Harlan, Oxford Laboratory Animal Centre, Bicester, UK) were acclimatized in a 12-hour night/day cycled laboratory (at 24°C) for at least one week. These animals were maintained on a standard pelleted diet (Labsure CRM Pellets; K & K Greef Ltd., Croydon, UK) with free access to water. They were then randomly assigned to groups, with 20 acting as controls and the remainder as test animals.

Rats within the control group were given a dose of corn oil (2.5 ml/kg body weight) via gastric intubation at 09.30 h whereas those within the test groups received the compounds under investigation as a suspension in corn oil (6.35 mmol/2.5 ml/kg body weight). One hour later (10.30 h) all animals received an intraperitoneal injection of [^{35}S]-L-cysteine (0.63 mmol/20 μCi/kg body weight). The rats were kept in separate metabolism cages ('Metabowls', Jencons Ltd., Herts, UK) and their 0-24 h urine collected directly into preweighed containers. Following collection and removal of the animals, the cages were thoroughly washed with aqueous ethanol (50% v/v).

Measurement of radioactivity

Aliquots of urine (0.1-1.0 ml) were added directly to scintillation fluid (20 ml; 'Ecoscint'; National Diagnostics, Atlanta, GA, USA) and counted by liquid scintillation spectrometry using a Packard Tri-Carb

4640 scintillation counter (Canberra-Packard Instruments, Paingbourne, Berkshire, UK) with internal standards being used for quench correction.

Quantification of inorganic sulphate

Aqueous sodium sulphate solution (1.8 ml, 2% w/v) was added to quadruplicate aliquots of urine (0.2 ml) followed by barium chloride (1 ml, 10% w/v) to two tubes and distilled water (1 ml) to the other two, which acted as controls. Aliquots (2 ml) of all the supernatants were then counted for radioactivity as above following vigorous vortex mixing and centrifugation. The difference obtained between the control and barium chloride treated tubes gave a measure of the precipitated inorganic sulphate originally within the urine. This radioactive precipitate itself was isolated, washed with water, and examined by chromatography (see below).

Any radioactive sulphate present in the organic (ethereal, bound) sulphate pool was determined as above after acid hydrolysis. Hydrochloric acid (2 ml, 7 M) was added to an aliquot of urine (1 ml) and water (4 ml) and boiled for 1 h. After cooling, the samples were neutralised with sodium hydroxide (~5 ml, 2.5 M) and then treated as above. This procedure enabled a measure of the total sulphate within the urine sample. Organic (ethereal, bound) sulphate was then calculated by difference.

Chromatography

Paper chromatography (Whatman 1; isobutyric acid - 1 M aq. NH_4OH, 5:3, v/v; descending method) was performed, together with standard $Na_2{}^{35}SO_4$ (Amersham International), and the dried papers scanned for radioactivity (Packard model 7200 radiochromatogram scanner, Packard Instruments Ltd., Berks, UK) and then sprayed with the potassium permanganate/barium chloride reagent which gives a diagnostic colouration for sulphate ions /13/. High-pressure liquid chromatography (HPLC) was also employed. The system used consisted of an LC-XPD pump and an LC-UV detector with a Phillips PU 4700 autoinjector and PM 8251 chart recorder (Pye Unicam, Cambridge, UK). The column (250 mm x 4 mm i.d.), a reversed phase Versapack C18 (Alltech Associates, Carnforth, Lancs), was attached to a guard column (20 mm x 4 mm i.d.) packed with octadecyl-bonded

glass beads (Pellicular Media, Greyhound Chromatography, Liverpool). The mobile phase was an aqueous solution of potassium hydrogen phthalate (1 mM) and tetrabutyl ammonium hydroxide (1 mM) adjusted to pH 6.1 with sodium hydroxide (0.5 M), being used with a flow rate of 1.5 ml/min. Sample injection size was 10 µl and ultra-violet detection occurred at 266 nm. Potassium hydrogen phthalate normally absorbs strongly at 266 nm but in the presence of sulphate it acts as a counter ion causing absorbance to be reduced and producing a negative peak, that may be inverted by reversing the recorder leads /14/.

RESULTS AND DISCUSSION

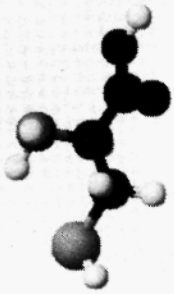
Radioactivity recovery

Results obtained from the group of control rats (n = 20) orally dosed with corn oil followed by an intraperitoneal injection of [^{35}S]-L-cysteine showed that 38.6 ± 8.8% of the radioactivity was recovered in the subsequent 0-24 h urine. This value was similar for the L-valine pre-treated animals (34.3 ± 14.2% dose), unlike the D-penicillamine (28.3 ± 7.4%) and *S*-carboxymethyl-L-cysteine (29.8 ± 9.3%) groups that showed a statistically significant decrease ($0.050>p>0.025$; Student's t-test) (Table 1).

Sulphate identification and quantification

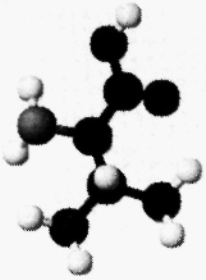
The radioactive precipitate recovered from urine samples following barium chloride treatment co-chromatographed with authentic sulphate and gave a characteristic pink spot on a buff ground (becoming white) when examined by paper chromatography and the potassium permanganate/barium chloride reagent /13/. In addition, co-chromatography of the radioactive component with authentic sulphate (Rt 6.2min) was achieved by HPLC /14/.

Virtually all of the urinary radioactivity could be accounted for as sulphate, with only a small amount (1.2 ± 1.0% dose, n = 40) in other unknown chemical forms. When compared with values obtained from control animals (37.3 ± 8.9% dose), the amount of radioactivity excreted as total sulphate from L-valine dosed rats showed no difference (33.9 ± 14.4% dose), whereas those pre-treated with



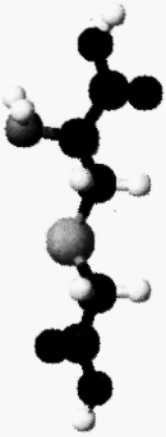
$HSCH_2CH(NH_2)COOH$

L-Cysteine



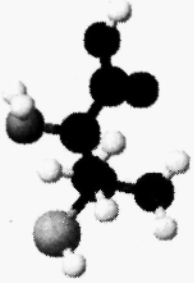
$(CH_3)_2CHCH(NH_2)COOH$

L-Valine



$HOOCCH_2SCH_2CH(NH_2)COOH$

S-Carboxymethyl-L-cysteine



$HSC(CH_3)_2CH(NH_2)COOH$

D-Penicillamine

Fig. 1: Comparison of the structures of the compounds used in this study.

D-penicillamine (27.6 ± 7.7% dose) or *S*-carboxymethyl-L-cysteine (28.4 ± 9.6% dose) displayed a statistically significant decrease ($0.050>p>0.025$; Student's t-test). This phenomenon could be traced to a statistically significant decrease in the excretion of inorganic sulphate following D-penicillamine (16.9 ± 6.6% dose versus 25.8 ± 7.5% dose) and *S*-carboxymethyl-L-cysteine (19.2 ± 8.5% dose versus 25.8 ± 7.5% dose) dosing ($0.050>p>0.025$; Student's t-test). In contrast, pre-treatment with L-valine had no measurable effects. There were no significant differences between any of the groups when organic (ethereal, bound) sulphate levels were compared (Table 1).

General discussion

The three compounds investigated in this study are all known to be rapidly absorbed from the gastrointestinal tract. In the rat, uptake of D-penicillamine has been reported to lie between 40% and 70% with over 50% being eliminated via the kidneys within 0-48 h /15-18/. *S*-Carboxymethyl-L-cysteine is also known to rapidly appear in tissues following oral administration /19,20/ with over 55% of the dose being excreted in 0-24 h urine /21,22/. The efficient gastrointestinal absorption of L-valine has led to its use as a marker for amino acid absorption and a test for alimentary function /23-25/.

Both D-penicillamine and *S*-carboxymethyl-L-cysteine are, in part, metabolically degraded to sulphate /9-12/ and it is possible that this extra sulphate being excreted via the kidneys could dilute the radioactive sulphate derived from the administered [^{35}S]-L-cysteine as the sulphate pool mixes. However, large variations in the amount of sulphate excreted in urine have been observed in the present study (from 27.3% to 57.2% [^{35}S]-L-cysteine dose as sulphate for control animals) and others /26-28/ suggesting abundant reserve capacity for excretion. In addition the excretion of sulphate via the kidney does not appear to be rate limiting /28-30/. It is not known where, within the complex sequence of events surrounding the degradation of cysteine to sulphate, that D-penicillamine or *S*-carboxymethyl-L-cysteine may interact, and additional investigations are required to throw light on this situation.

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