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STUDIES ON D-PENICILLAMINE METABOLISM IN CYSTINURIA AND RHEUMATOID ARTHRITIS: ISOLATION OF *S*-METHYL-D-PENICILLAMINE

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Abstract An unknown metabolite of D-penicillamine found in the urine of patients receiving the drug has been isolated by ion-exchange chromatography. It was identified as *S*-methyl-D-penicillamine by mass spectrometry and its structure was confirmed by synthesis.

In subjects receiving D-penicillamine for treatment of cystinuria and rheumatoid arthritis 4 and 8 per cent respectively of the dose was methylated. The total percentage of the D-penicillamine dose excreted (as cysteine-penicillamine plus penicillamine disulphide plus *S*-methyl-D-penicillamine) was 40 and 34 per cent in cystinuria and rheumatoid arthritis respectively. The findings in two cases of Wilson's Disease were similar to those in rheumatoid arthritis.

In studies on four cystinuria patients an average of 12 per cent of the administered D-penicillamine was recovered in the faeces mainly as penicillamine disulphide. However, only between 42 and 53 per cent of the dose was identified in the urine and faeces, so that approximately 50 per cent of the administered drug was unaccounted for.

D-Penicillamine caused a 32 per cent reduction in the urinary excretion of cysteine residues in cystinuria but a 400 per cent increase in their excretion in rheumatoid arthritis.

Since 1956 penicillamine ($\beta\beta$ dimethylcysteine) has been extensively employed in the treatment of Wilson's Disease [1]; and in 1963 it was also introduced for the treatment of cystinuria [2]. A recent controlled study [3] has confirmed its beneficial effects in the treatment of rheumatoid arthritis [4]. A number of other therapeutic effects of penicillamine have been reported [5]. *In vitro* effects reported in the literature include inhibition of DNA and protein synthesis [5], selective inhibition of polio virus growth [6] and prevention of collagen cross linking [7]. The biochemical actions of penicillamine are usually understood either in terms of its metal chelating properties, its ability to undergo thiol exchange [8], or inhibition of pyridoxal metabolism by thiazolidine formation [9, 10]. These biochemical and *in vitro* effects have been implicated in the clinically observed side effects of penicillamine therapy including proteinuria [11], thrombocytopenia [12] and loss of taste [13].

Early work on the metabolism and toxicity [14] of the D and L forms of penicillamine indicated the much greater toxicity of the L-form and therefore recent clinical studies have employed the natural isomer, D-penicillamine. Only recently have studies on the pharmacology of D-penicillamine been performed. Gibbs and Walshe [15] studied the fate of orally administered [^{35}S]DL-penicillamine in six cases of Wilson's Disease and Wass and Evered have studied the intestinal absorption of L and D-penicillamine in the rat [16]. No metabolites of D-penicillamine other than the mixed disulphide with cysteine and its internal disulphide [2] had been identified. In cystinuria these disulphides account for only one quarter to one half of the administered D-penicillamine [17]; a similar finding has been reported in normal adults [18]. Because D-penicillamine is administered in doses of up to 3 g/day for long periods, possibly lifetimes, a more detailed understanding of the metabolism is desirable. In an attempt to uncover possible metabolites, the following study of penicillamine metabolism in patients with cystinuria, rheumatoid arthritis and Wilson's Disease was undertaken.

Examination of the urinary amino acid chromatograms, from cystinuric patients, revealed the presence of an unknown peak in the alanine region. This peak was observed to vary with the D-penicillamine dosage and so the isolation and quantitation of this unknown was undertaken.

Subjects. Sixteen patients with cystinuria, five with rheumatoid arthritis and two with Wilson's Disease were included in the study. All had been receiving D-penicillamine therapy for at least one month.

Specimens. All 24 hr urines were collected into 20 ml concentrated hydrochloric acid to prevent further disulphide exchange. Fasting early morning plasmas for amino acid analysis were prepared using solid salicylsulphonic acid as protein precipitant and nor-leucine as an internal standard.

Balance studies. Faecal and 24 hr urine collections were made for a period of at least 5 days on four cystinuric subjects receiving D-penicillamine. The individual faecal collections were immediately deep frozen until the end of the study. Faecal specimens were then pooled and slurried with approximately twice their volume of water for 2 hr with a mechanical stirrer. The total weight of the slurry was measured and triplicate weighed aliquots (approx 2 g) were homogenised with 1 ml of 0·1 M HCl. The homogenate was then extracted with acetone, centrifuged

to remove the debris and the volume of the supernatant measured [19]. Appropriate aliquots were applied direct to the amino acid analyser.

MATERIALS

D-Penicillamine used throughout this study was supplied either as the free base or the hydrochloride (Distamine; Dista Products Ltd., Liverpool) and was found to be chromatographically pure.

Performic acid was prepared by mixing 1 part of hydrogen peroxide (100 vol) with 10 parts of 98 per cent formic acid. The mixture was kept at room temperature for 24 hr. Two parts of this mixture was added to 1 part of the solution to be oxidised and left at 4° for 3 hr. The mixture was evaporated to dryness before further analysis.

Cysteine-penicillamine disulphide and penicillamine disulphide were synthesised by the methods of Crawhall *et al.* [20]; Clarke *et al.* [21] respectively.

All chemicals were analytical grade reagents.

METHODS

Amino acid analysis was performed on a Technicon NC-1 analyser using a 140 × 0·63 cm column of Chromobeads Type B and the buffer system of Purdie *et al.* [22] at a flow rate of 40 ml hr^{-1}. Although this system gave good separation of cystine, cysteine-penicillamine disulphide and penicillamine disulphide, it did not resolve alanine from *S*-methyl penicillamine. A rapid system was therefore developed with improved resolution of *S*-methyl-penicillamine, which enabled quantitative measurements to be completed in about 1 hr. A 22 × 0·63 cm Pyrex column maintained at 60 ± 0·5° was filled to a depth of 18 cm with Chromobeads type B and eluted at 40 ml hr^{-1} with either (a) 0·067 M tri-sodium citrate buffer pH 2·65 for quantitative studies or (b) a volatile buffer consisting of 0·1 M pyridine titrated to pH 2·65 with 98 per cent formic acid for preparative separations. When required the column eluant was monitored at 264 nm using a Cecil CE212 variable wavelength u.v. monitor equipped with a 75 μl 1 cm silica flow cell, prior to entry into the ninhydrin-analytical system.

High voltage electrophoresis was performed on a Miles Hivolt (Shoreham, Sussex) model 2 unit using Whatman 3 MM paper in 8 per cent (v/v) formic acid at 4 kV for 30 min per paper. U.V. spectra were recorded on a Unicam SP 800 spectrophotometer.

Mass spectrometry. Spectra were obtained on either a Varian MAT model SM1 or M86 mass spectrometer. The operating conditions for both instruments were 70 V electron energy, 300 μA electron current sample vaporization and in source temperatures 150°. The atomic compositions (given in the figures) were obtained by accurate (±0·003 AMU) mass measurements of the appropriate ions using the double focussing mass spectrometer (SM1) at a revolving power of 10,000 with perfluorokerosine as reference material. The difference between the calculated and mean experimental masses for each ion did not exceed 0·002 AMU.

Table 1. Elution times from 140 cm column*

Amino acid	Elution time (min)
Glycine	274
Alanine	306
S-methyl-penicillamine	316
Cystine	426
Cysteine-penicillamine disulphide	444
Penicillamine disulphide	472
Nor-leucine	530

* Using gradient of Purdie *et al.* [22].

EXPERIMENTAL

Inspection of a number of amino acid chromatograms of urine from patients receiving D-penicillamine showed the presence of a ninhydrin positive peak not resolved from alanine (Table 1).

As shown in Fig. 1, using the short column system it was possible to resolve the unknown from alanine. Initial tests to identify the unknown by simple procedures followed by short column chromatography are listed below. When the elutant from either column was monitored at 264 nm a small peak was found to coincide with the ninhydrin peak due to the unknown. Similar peaks occurred for cystine, cysteine-penicillamine mixed disulphide and penicillamine disulphide.

(a) Urine was co-chromatographed with 0·2 μmole of freshly prepared D-penicillamine, which eluted at 50 min, 12 min before the unknown.

(b) The possibility that the unknown substance formed during storage of the urines was investigated by analysis of freshly voided cystinuric urine. The unknown was found to be present and its size did not change after several days at room temperature.

(c) No peak in the unknown's position was found in the urine of five cystinuric patients who were not receiving D-penicillamine. When these patients received D-penicillamine they excreted the unknown.

(d) In order to determine if the chemical instability of D-penicillamine in physiological fluids could produce the unknown substance D-penicillamine (2 m-mole/L) was incubated for 8 hr at 37° with freshly voided urine from a cystinuric patient not receiving penicillamine. No peak formed in the region of the unknown. A similar experiment with fresh plasma was also negative.

(e) Performic oxidation of urine resulted in oxidation of the unknown substance. When the chromatographic fraction containing the unknown was subjected to performic oxidation, a new frontally eluted peak in the position of penicillamine sulphonic acid appeared.

(f) The chromatographic fraction containing the unknown was found to give a negative nitroprusside test when performed both with and without reduction with cyanide. The presence of a free thiol group was therefore excluded but since penicillamine containing disulphides are resistant to reduction [23] a disulphide could not be excluded.

Isolation of unknown. Three litres of acidified urine from a patient found to have an above average excretion of the unknown was filtered and then desalted on a 2 × 100 cm Dowex 50 × 8 (H^+ form) column. The column was washed with 2 litres of water and then eluted with 1 M ammonium hydroxide, which was collected until the eluant no longer gave a positive nitroprusside test. The elutant was reduced to

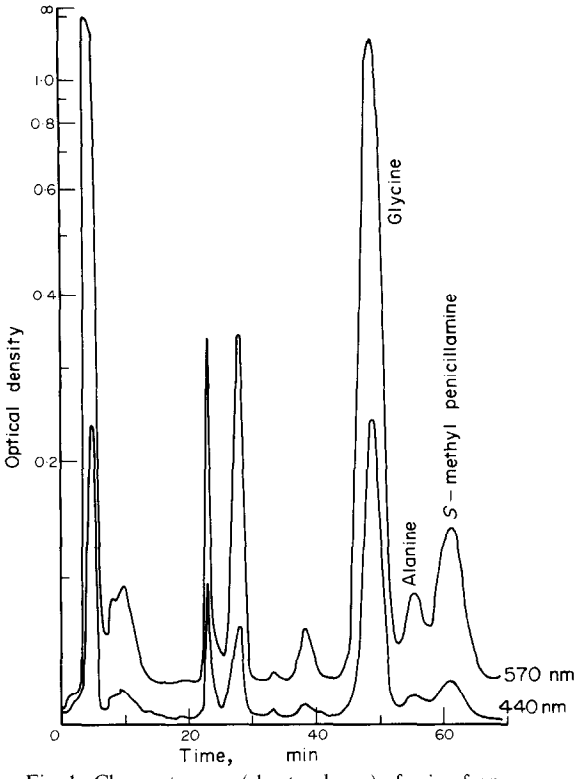

Fig. 1. Chromatogram (short column) of urine from a cystinuric subject receiving D-penicillamine.

dryness on a rotary evaporator. The residue was dissolved on 100 ml of 0·1 M HCl and filtered. Amino acid analysis of the filtrate showed the presence of the unknown plus other acidic and neutral amino acids.

1·5 ml of the filtrate was applied to the 22 cm analyser column equilibrated with pyridine–formic buffer and eluted as previously described. The column elutant was split so that only 1 ml/hr entered the analytical system and 5 min fractions of the remainder were collected on a variable time-based fraction collector. Using the pyridine system the unknown eluted immediately prior to glycine. Because of the highly concentrated nature of the samples it was necessary to clean the column by pumping 0·5 M NaOH for 15 min before re-equilibration to prevent the contamination of subsequent runs. In all 25 ml of the urine concentrate (equivalent to 750 ml of original urine) were separated in this manner.

The pooled fractions were reduced to dryness and dissolved in 10 ml of 8 per cent formic acid. Amino acid analysis showed that the unknown was contaminated with a large amount of glycine and some alanine.

The unknown was separated from glycine and alanine by high voltage electrophoresis. 0·5 ml of the solution of the unknown were applied per paper and two papers were electrophoresed simultaneously. Marker strips were stained with ninhydrin and the band containing the unknown removed. The strips were eluted with 0·01 M HCl by descending chromatography. The elutants were pooled, evaporated to dryness and re-chromatographed on the 22 cm column with pyridine–formic buffer to remove final traces of ninhydrin positive impurities. The fractions were collected and desalted by absorption onto a 0·6 × 5 cm column of Zeocarb 225 (200–400 mesh H^+ form) washing with water (5 × 1 ml) and elution with 1 M ammonium hydroxide. Approximately 10 mg of a slightly off-white solid were obtained on lyophilizing the elutant.

The u.v. spectra of the isolated compound exhibited three weak absorption maxima at 257, 264 and 271 nm.

The mass spectra of the metabolite is shown in Fig. 2b together with that of penicillamine (Fig. 2a). Comparison of the two showed a rather similar pattern of decomposition. However, the molecular ion and certain prominent fragments, denoted a, b, c, have masses 14 AMU greater in the spectrum of the metabolite than in penicillamine. (The elemental comparison of these ions, indicated in the figure, show that these mass differences correspond to CH_2, indicating the metabolite to be a penicillamine homologue.) The location of the extra carbon atom on the sulphur is indicated by the prominent ions c and d attributed to $(CH_2)_2C = SCH_3$ + (m/e 89) and CH_3SH_2 + (m/e 49) respectively in the spectrum of the metabolite. Furthermore, the molecular ion of the metabolite loses a CH_3S moiety to yield an ion of m/e 116. An ion of the same mass is obtained by loss of the HS group from penicillamine but there is no similar loss of HS from M^+ of the metabolite to give an ion of m/e 130.

*Synthesis of S-methyl-*D*-penicillamine.* To a solution of 500 mg D-penicillamine in 50 ml of anhydrous liquid ammonia, stirred by a strong mechanical stirrer, was added small portions of clean sodium metal until the blue coloration persisted for a few minutes.

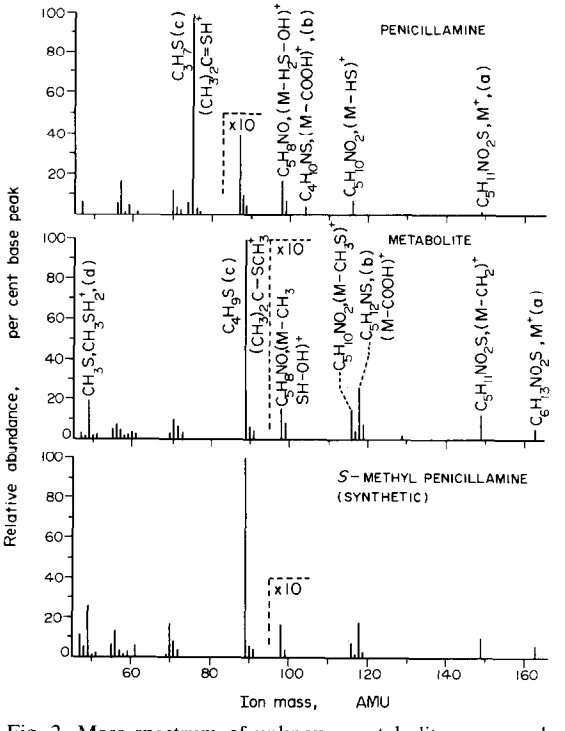

Fig. 2. Mass spectrum of unknown metabolite compared with that of D-penicillamine and synthetic *S*-methyl-D-penicillamine.

Table 2. Urinary excretion of penicillamine metabolites

Subject	Dose μmole/24 hr	$PSCH_3$ μmole/24 hr	C-P μmole/24 hr	P_2 μmole/24 hr	% of dose excreted
Cystinuria					
1	7300	178	1880	545	43
2	7300	243	1445	375	34
3	8065	276	2020	550	42
4	8920	226	2260	554	40
5	9670	108	1520	440	26
6	9670	403	2690	476	42
7	9670	460	2500	755	46
8	10000	348	1900	296	28
9	12200	221	3470	950	46
10	13300	306	3180	595	35
11	15000	1374	4260	1955	64
12	15000	510	3350	850	37
13	16780	810	3920	1060	41
14	20130	1130	5260	1910	51
15	20130	750	3470	1220	33
Rheumatoid arthritis					
16	4030	408	820	330	47
17	5030	305	930	400	40
18	5030	184	393	122	20
19	6700	430	720	532	33
20	13000	541	1890	826	32
Wilson's Disease					
21	21300	1100	3240	3430	53
22	10640	147	1380	880	31

Key—$PSCH_3$, *S*-methyl-D-penicillamine; C-P, cysteine-penicillamine disulphide; P_2, penicillamine disulphide.

Methyl iodide (approx 0·4 ml) was slowly added until the nitroprusside test on a small aliquot was negative. The reaction mixture was reduced to dryness at 37° on a rotary evaporator. The residue was dissolved in 20 ml of deionised water, neutralised with hydrochloric acid and reduced to dryness again. Ion-exchange chromatography showed the sample to contain a major peak as well as a number of other components including penicillamine disulphide.

The residue was dissolved in 0·1 M HCl, and absorbed onto a 8 × 2 cm column of Zeocarb 225 resin (H^+ form 200–400 mesh), washed with 150 ml of water and then eluted with pH 2·65 pyridine/formic acid buffer. The product eluted between 50 and 150 ml. The elutant was reduced to dryness under vacuum. The u.v. spectra indicated the possible presence of a formate salt. The product was desalted by passage down a 5 × 0·6 cm cation column as described under the isolation procedure. The product could not be crystallized but it was found to give only one ninhydrin positive spot by TLC chromatography. Yield 200 mg.

Comparison of synthetic and isolated S-*methyl*-D-*penicillamine.* Samples of synthetic and isolated *S*-methyl-D-penicillamine were chromatographed both singly and together by the short column system using both citrate and pyridine–formic buffers. In both systems the elution times and 570 nm/440 nm ratios were identical.

The two compounds also co-chromatographed on cellulose thin layer plates in the following solvent systems: Butan-1-ol–acetic acid–water (60:15:25) and Butan-1-ol–acetone–diethylamine–water (70:70:14:35) [24]. The R_f values were 0·60 and 0·66 respectively, values similar to those obtained for methionine. High voltage electrophoresis in 8 per cent formic acid was also unable to separate the two compounds.

The mass spectra of the synthetic *S*-methyl-penicillamine was identical with that obtained for the isolated unknown (Fig. 2).

RESULTS

The isolation and identification of the unknown metabolite of D-penicillamine as *S*-methyl-D-penicillamine and the subsequent synthesis of the pure compound have enabled the quantitation of the urinary excretion of this metabolite in patients receiving penicillamine.

S-methyl-D-penicillamine could only be detected in some plasma samples as a small peak poorly separated from the large alanine peak but the amounts were below the level of quantitation (i.e. less than 1 μmole/100 ml) using the amino acid analyser. No *S*-methyl-D-penicillamine could be detected in the faeces of cystinuric subjects during the balance studies.

The urinary excretion of penicillamine metabolites is detailed in Table 2 for a number of patients receiving between 250 mg and 3 g of D-penicillamine per day.

For the cystinuric patients the urinary excretion of *S*-methyl-D-penicillamine accounted for between 0·5 and 10 per cent of the penicillamine dose with a mean of about 4 per cent. The amount of this metabolite excreted in the urine increased linearly with dosage

and was independent of whether the drug was supplied as the free base or the hydrochloride. In serial studies on two cystinuric subjects the percentage of the penicillamine dose excreted varied by less than 5 per cent in both cases over a 5-day period. In the same period *S*-methyl-D-penicillamine comprised between 4 and 16 per cent of the metabolised penicillamine found in the urine of the two subjects. The percentage of the penicillamine dose methylated by the rheumatoid patients was higher than for cystinurics 6 to 12 per cent with a mean of about 8 per cent. Cysteine–penicillamine disulphide was the major excretion product with some penicillamine disulphide also excreted in the rheumatoid patients who were receiving comparatively low doses of D-penicillamine. *S*-methyl-D-penicillamine and the two disulphides were also found in the urine of four patients with Wilson's Disease receiving the drug.

The percentage of the penicillamine dose excreted in the urine as the *S*-methyl derivative plus the disulphides varied from 26 to 64 per cent (40 ± 11%, mean ± ISD, n = 16) for the cystinurics and from 20 to 47 per cent (mean 34%, n = 5) for the rheumatoid patients. Similar values were obtained in two cases of Wilson's Disease. No free penicillamine could be detected in fresh urine samples from patients with either cystinuria or rheumatoid arthritis.

The results of the balance studies on four cystinuric patients are given in Table 3. Between 26 and 44 per cent of the administered penicillamine was detected in metabolised form in the urine. A further 4–12 per cent of the dose was not absorbed by the intestinal tract and was found in the faeces mainly as penicillamine disulphide. Only trace quantities of cysteine–penicillamine could be detected in faeces homogenised in acid. This increased with alkaline extraction but total recovery of penicillamine was reduced by 10 per cent. The total percentage of the dose found in faeces plus urine varied from 42 to 53 per cent. In ten of the cystinuric subjects where cystine excretion before treatment with D-penicillamine was known accurately the excretion of cysteine residues (i.e. as cystine and cysteine-penicillamine disulphide) was only 67·6 ± 15·4% (mean ± 1SD) of the original cystine excretion which had been 3600 ± 880 μmole/24 hr. On the other hand penicillamine administration to the five patients with rheumatoid arthritis led to an increased excretion of cysteine moieties. Although cystine excretion was reduced to 35 ± 10 μmole/24 hr compared to normal values of 120 ± 60 μmole/24 hr, the excretion of cysteine moieties increased to 1020 ± 560 μmole/24 hr equivalent to 510 μmole of cystine. Similar values were obtained in two cases of Wilson's Disease.

DISCUSSION

Although D-penicillamine is being used increasingly as a therapeutic agent relatively little information on its metabolism in man is available. Previous studies have shown cysteine–penicillamine disulphide and penicillamine disulphide to be the main urinary excretion products in cystinuria. The present study confirms the excretion of these disulphides in both patients with Wilson's Disease and rheumatoid arthritis receiving the drug and has also isolated and identified a further metabolite *S*-methyl-D-penicillamine. Gibbs and Walshe [15] using ^{35}S labelled DL penicillamine identified by two dimensional chromatography followed by auto-radiography the mixed disulphide and four other trace metabolites in the urine of patients with Wilson's Disease receiving D-penicillamine. It is possible that one of their unidentified metabolites was *S*-methyl-D-penicillamine. No previous report of this metabolite could be found in the literature. This is probably because its resolution from glycine and alanine on an amino acid analyser is difficult due to its elution time varying considerably with only small changes in pH and ionic strength of the buffers. The use of low resolution resins or rapid elution systems would probably result in the *S*-methyl-penicillamine peak eluting with the large glycine peak in urine. An examination of a chromatogram of urine from a cystinuric subject receiving D-penicillamine given by Fowler and Robins (Fig. 5) [25], when monitoring both with ninhydrin and an iodoplatinate reagent which specifically detects sulphur containing compounds, shows an unidentified peak on the iodoplatinate trace lying under the glycine peak on the ninhydrin trace. It is probable that this peak was *S*-methyl penicillamine.

Biological methylation of D-penicillamine could occur during the conversion of methionine to homocysteine via *S*-adenosyl-methionine (SAM). The methylation of foreign thiols such as mercaptoethanol by

Table 3. Faecal and urinary excretion of D-penicillamine in cystinuria

Subject	Days of study	Total dose of penicillamine given (μmole)	Total excretion of metabolites (μmole) $PSCH_3$	C-P	P_2	% of dose excreted
(a) Faecal excretion of penicillamine metabolites						
CO	5	76,500 (base)	*	*	1520	4
RB	7	17,000 (HCl)	*	*	1350	16
EL	6	100,000 (base)	*	370	7600	16
JO	5	76,500 (base)	*	*	4500	12
(b) Urinary excretion of penicillamine metabolites						
CO	5	76,500 (base)	3460	13300	7160	44
RB	7	17,000 (HCl)	450	2500	700	26
EL	6	100,000 (base)	3580	14750	5500	29
JO	5	76,500 (base)	2950	19300	4300	41

* Not detectable.

SAM has been shown to be catalysed by microsomes from various tissues [26]. The *S*-methyl-penicillamine formed appeared to be cleared from the blood at a high rate although the low blood levels prevented the calculation of a clearance value.

Although *S*-methyl penicillamine accounted for more than 10 per cent of the penicillamine moiety found in the urine of patients receiving the drug, there was still a large deficit of recovered urinary penicillamine over the dose. The average total urinary excretion was less than 50 per cent of the dose. Gibbs and Walshe [16] found that about 75 per cent of the ^{35}S label had appeared in the urine in 48 hr. Because their study employed the DL isomer interpretation of their results in terms of the D-isomer alone is difficult. Not only is the L isomer metabolised differently [14] but recent studies in the rat have demonstrated both active transport [16] and nearly complete intestinal absorption [27] of this isomer. Therefore if 100 per cent of the [^{35}S]L-isomer was absorbed in man only 50 per cent absorption of the D-isomer would be needed to give a mean 75 per cent absorption. Using [^{14}C]D-penicillamine Ruiz-Torres and Kurten [27] found 50 per cent absorption from the rat intestine following an oral dose.

In the present study less than 20 per cent of the D-penicillamine dose was recovered in the faeces as identifiable penicillamine metabolites, mainly the internal disulphide. Because our balance studies were performed in cystinuric subjects, who have impaired active transport of cystine but not cysteine in the gut, it is possible that any penicillamine disulphide formed would not be readily absorbed whereas free penicillamine is absorbed. Therefore the faecal levels could be higher in cystinuria than in either Wilson's Disease or rheumatoid arthritis but as no cystine was found in the cystinuric faeces substantial passive absorption of disulphides must occur.

Some 30 per cent of the dose was not detected either in the urine or faeces as known penicillamine metabolites. It is possible that bacterial degradation of penicillamine in the gut could occur forming sulphate which would not have been detected during the present studies. But until detailed studies with labelled D-penicillamine can be performed, the manner in which this unaccounted third of the dose is excreted cannot be explained.

Crawhall and Thompson [17] suggested that the increased excretion of cysteine residues in Wilson's Disease was because the unnatural mixed disulphide, unlike cystine, would probably not be reabsorbed by the renal tubule and would therefore be cleared from the blood at a greater rate. The same explanation should also apply to the similar findings in patients with rheumatoid arthritis.

The 30 per cent reduction in the urinary excretion of cysteine residues by cystinurics receiving D-penicillamine, observed in the present study is in agreement with the results of Crawhall and Thompson [17] and Bartter *et al.* [28]. Stokes *et al.* [29] suggested that the effect was due to some unidentified effect of penicillamine on cyst(e)ine metabolism.

As the interconversion of methionine via SAM to homocysteine and finally cysteine is a possible site for the methylation of penicillamine it is probable that, as a result, inhibition of cysteine synthesis could occur. Although other possible mechanisms for this inhibition would be (a) disulphide formation with naturally occurring thiols, e.g. homocysteine and glutathione, (b) binding of D-penicillamine via disulphide bonds to proteins and (c) thiazolidine formation with carbonyl groups. All of these could possibly cause inhibition of cysteine synthesis.

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