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# TRANSPORT OF PENICILLAMINE ACROSS MUCOSA OF THE RAT SMALL INTESTINE *IN VITRO*

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**Abstract**—Transport of D- and L-penicillamine against a concentration gradient was studied using everted sacs of rat small intestine. Uptake by segments of intestinal tissue was also studied. The L-isomer was actively transported but the D-isomer was not. Active transport of L-penicillamine was decreased in the presence of cyanide, L-methionine and L-valine. The effect of both penicillamine isomers on the transport of $^{35}$S-L-cysteine was also investigated.

TRANSPORT of many amino acid analogues has been studied using mammalian small intestine. These analogues may have pharmacological or therapeutic properties. Some of these compounds exhibit active transport against a concentration gradient, as for instance some amino acid derivatives of nitrogen mustards,[1] and 1-aminocyclopentane-1-carboxylic acid.[2] Other amino acid analogues may pass across the mucosa by passive diffusion with the concentration gradient, as for example $\epsilon$-aminocaproic acid,[2] and some other $\omega$-amino acids.[3]

Penicillamine ($\beta,\beta$-dimethylcysteine or $\beta$-mercaptovaline) is used therapeutically. For example, doses of up to 1 g/day are used in treating Wilson's disease,[4] cystinuria,[5] lead poisoning[6] and rheumatoid arthritis.[7]

Possible toxic effects of penicillamine should be considered, since a few patients have been found to develop the nephrotoxic syndrome.[6, 8–10] It was therefore of interest to study the mode of transport of penicillamine, since it might compete with amino acids for absorption from the intestine,[11] reabsorption from the renal tubule, or transport across the cell membrane.

L- but not D-penicillamine has proved to be toxic to rats.[12, 13] There is evidence that penicillamine is an antagonist of pyridoxal-5'-phosphate (PLP), a member of the vitamin $B_6$ group.[14–16, 20–22] The activity of two PLP-dependent enzymes was decreased in the tissues of rats treated with L-penicillamine, but parallel studies with D-penicillamine were not made.[14] Such experiments were quoted to support claims that only L-penicillamine is a PLP-antagonist.[15, 16] However, PLP forms stable thiazolidines with both D- and L-isomers of penicillamine.[14, 17] Furthermore, the stability constants of the thiazolidines formed between PLP and the penicillamine isomers (D,L and DL) are numerically identical.[18] For that reason thiazolidine formation *per se* cannot account for the different toxicities of two penicillamine isomers.

The hypothesis that the therapeutic agent D-penicillamine is also, like the L-isomer, a vitamin $B_6$-antagonist,[19] has now received experimental support, both in rats[20] and in the human.[21, 22] Pyridoxine supplements are now recommended during penicillamine

therapy.[23] Formerly the synthetic DL-isomer was used therapeutically in the United States, but only D-penicillamine, from hydrolysis of penicillin, was used in this country.[23] Walshe[24] considers that pyridoxine supplements are necessary when DL-isomer is used, but are unnecessary with the D-isomer when administered in doses below 40 mg/kg body wt./day.

In the present study we have investigated the transport of penicillamine isomers across the intestinal mucosa of the rat *in vitro*. The uptake of both isomers by segments of small intestine was also studied.

## EXPERIMENTAL

### *Animals*

Male albino rats of the Wistar strain, 200–300 g body weight, were starved overnight but given drinking water *ad lib.*

### *Preparation of everted intestinal sacs*

The eversion of the intestine was carried out as described previously.[1] Test solutions were made in Krebs phosphate saline (pH 7·4) containing 0·3% glucose but no calcium salts[25] and were saturated with 5% $CO_2$/95% $O_2$. Each sac contained 0·4 ml test solution. Two sacs were immersed in 15 ml of test solution in a 50-ml conical flask, gassed again for 2 min with 5% $CO_2$/95% $O_2$, and shaken at 37° for 1 hr. Generally, two sacs only were taken from a single animal, starting from the proximal jejunum. In most experiments with inhibitors, or in experiments designed to compare the effect of two isomers, four or more consecutive sacs were taken from one animal. At the start of the experiment the same test solution was present both inside the sac (serosal fluid) and outside the sac (mucosal fluid).

### *Tissue uptake*

The preparation of the segments of the rat small intestine were carried out according to Agar *et al.*[26] The uptake of penicillamine isomers was measured with an incubation period of 4 min.[27]

### *Test compounds*

D-, DL- and L-penicillamine used were commercial samples or gifts. D-isomer was obtained from Dista Products Ltd., L-isomer from Calbiochem Ltd., and Mann Research Laboratories, and DL-isomer from Sigma Chemical Co. Purity was checked either by high-voltage electrophoresis (Locarte and Co. Ltd.), after preliminary reaction with *N*-ethylmaleimide to block the thiol group, on Whatman 3 MM filter paper, in formic–acetic acid buffer (1·5 M formic acid + 2 M acetic acid, 1:1 by volume), pH 2·0, at 100 mA, for 2 hr, or by estimation of the thiol content, before and after reduction. In all samples of penicillamine the content of the oxidized form was never above 10% of the total. $^{35}$S-L-Cysteine was purchased from the Radiochemical Centre (Amersham, England).

### *Analytical methods*

After incubation the serosal and mucosal fluids were deproteinised by treatment with 24 volumes of 3% aqueous sulphosalicylic acid. The standard solutions were treated similarly. The deproteinised samples were analysed for thiol content using Ellman's

reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB).[28] This reagent reacts with thiols to undergo a disulphide exchange reaction, yielding a mixed disulphide and the reduced reagent. When fully dissociated the latter exhibits a deeper colour than the corresponding disulphide, and can be estimated by measuring the absorption at 410 mμ. The determination was carried out using a modified method,[29] by adding 4·0 ml of $0{\cdot}5 \times 10^{-3}$ M DTNB in 1·0 M phosphate buffer (pH 7·0) to 1 ml of deproteinized sample. The extinction at 410 mμ was then read immediately in a Unicam SP 1400 spectrophotometer. The colour faded slightly on standing, but extrapolation to zero time was not necessary. The above procedure gave the reduced thiol content of the sample. By electrolytic reduction of a further aliquot of deproteinized sample,[30] followed by the procedure outlined above, the total thiol content was obtained. The DTNB method was sensitive to as little as 0·02 μmoles of a thiol compound, and the colour formation was linear over the range 0·02 to 0·25 μmoles.

In experiments with tissue segments, samples of the medium after removal of tissue were analysed by the procedure described above, using the DTNB reagent. Blanks consisted of tissue segments incubated in Krebs phosphate saline at pH 7·4.

In everted-sac experiments designed to measure the transport of $^{35}$S-L-cysteine the samples of the serosal and the mucosal fluids were deproteinized after incubation by immersing the centrifuge tubes in boiling water for 10 min and, after centrifuging, 0·1 ml aliquots of clear supernatant solution were added to 5 ml scintillating fluid for radiochemical assay. The scintillating fluid consisted of 6·8 g Butyl PBD [2-(4′-t-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole], 80 g naphthalene, 600 ml toluene, and 400 ml methyl Cellosolve. Radioactivity measurements were carried out in a PANAX liquid scintillation counter. 10,000 counts were recorded for each sample duplicate.

Experiments with $^{35}$S-L-cysteine were carried out in the presence of Cleland's reagent (dithiothreitol).[31] Because of its low redox potential (−0·33 V at pH 7·0) dithiothreitol is capable of maintaining monothiols completely in the reduced state and of preventing the formation of disulphides.[32] This was tested by submitting a solution of 0·1 mM $^{35}$S-L-cysteine and 2·5 mM D-penicillamine in Krebs phosphate saline (pH 7·4) containing 2 mM dithiothreitol to conditions simulating those in experiments with everted sacs. The radioactivity of this solution was, however, much greater (600 c/sec/50 μl). After 1 hr incubation at 37° in an atmosphere of oxygen 1 ml solution was added to 1 ml 100 mM solution of *N*-ethylmaleimide in phosphate buffer (pH 7·4) to block the oxidation of thiols.[33] The resulting solution (50 μl) was subjected to two-dimensional chromatography using *tert*-butanol–formic acid–water (70:15:15) in the first direction and acetone–ethyl methyl ketone–water (40:40:20) in the second direction. A mixture of cystine, penicillamine disulphide, and mixed cysteine–penicillamine disulphide was chromatographed similarly, and used as a map. After drying the chromatograms were cut into sections which were placed in counting vials containing 5 ml scintillating liquid. No radioactivity was found in the area corresponding to disulphides.

*Treatment of tissue*

The physiological state of the tissue was assessed after the experiment from the water content and from histological examination. The everted sacs were drained and dried overnight at 105°. Fat was removed from dry tissue by multiple extractions with

petroleum ether and ether.[34] For histology appropriate sacs were drained, fixed in Heidenhain's Susa fixative and sections stained with Weigert's haematoxylin stain, using chromotrope as counterstain.

*Water transport*

Transport of water by the everted sac of rat small intestine was studied by including 6% (w/v) inulin in the test solutions, and measuring the dilution of inulin in the serosal fluid. Eight sacs were taken from each animal starting from proximal jejunum, and filled respectively with 0·4 ml Krebs phosphate saline, L-penicillamine, D-penicillamine, or L-valine in phosphate saline, repeating the procedure twice along the gut. Solutions in flasks were identical to those used for filling the sacs. Inulin was determined using the method of Roe *et al.*[35]

*Injection with pyridoxine*

Three hours before the experiment, each animal was injected intraperitoneally with 1 ml of a 0·3% (w/v) solution of pyridoxine hydrochloride in 0·9% NaCl.

## RESULTS

The concentration ratio was calculated as the fraction:

$$\frac{\text{Concentration of penicillamine in the serosal fluid}}{\text{Concentration of penicillamine in the mucosal fluid}}.$$

Both concentrations were expressed as values after reduction, i.e. as total thiol. A ratio greater than 1·0 indicated active transport against a concentration gradient. The rate of transfer to the serosal fluid was calculated by subtracting the amount originally placed in the sac from that found in the sac by analysis. Since a semi-specific method was employed for determining penicillamine, blank controls were carried out using phosphate saline, with or without added glycine (5 mM). In blank experiments the concentration of thiols in the mucosal fluid was generally about 0·1 mM whilst in the serosal fluid it ranged from 0·3 to 0·6 mM. Corrections for endogenous thiol were not applied, since they were variable. For that reason both the concentration ratios and the rates of uptake reported here have no absolute value, but are comparative.

The expression of concentration gradients and the rates of uptake by means of values estimated after reduction was adopted because in our early experiments relatively large proportions of penicillamine in the serosal fluid were found to be in the disulphide form. However, this was later found to be due to the contact of penicillamine with the metal syringe and the hypodermic needle used in filling the sacs. When plastic or glass equipment was used, the catalytic oxidation in the serosal fluid was considerably reduced. Generally, the content of penicillamine disulphide in the serosal fluid was only slightly higher than it was in the standard solution. The uptake of penicillamine by the tissue segments was expressed as the loss of penicillamine as total thiol (value after reduction) from the incubation medium. The uptake was corrected for the total thiol released into the medium by segments incubated in Krebs phosphate saline at pH 7·4.

The water content of the sacs was reasonably constant (Tables 1, 2, 3 and 7). Sacs submitted to different treatments (e.g. phosphate saline, D- or L-penicillamine at different concentrations) were indistinguishable in their water content as well as on

TABLE 1. THE EFFECT OF CONCENTRATION ON THE ACCUMULATION OF PENICILLAMINE ISOMERS BY EVERTED SACS OF RAT SMALL INTESTINE

| Isomer | Concentration (mM) | Concentration ratio* (Mean ± S.E.M.†) | Rate of transference (μM/g fat-free dry wt.) Mean ± S.E.M. | Water content (g/100 g fat-free fresh tissue) Mean |
|---|---|---|---|---|
| D | 1·25 | 1·00 ± 0·03 (6) | — | 87·0 |
| D | 2·5 | 0·84 ± 0·03 (6) | — | 87·7 |
| D | 5 | 0·82 ± 0·02 (12) | — | 87·4 |
| DL | 2·5 | 1·13 ± 0·03 (6) | — | 87·7 |
| DL | 5 | 1·04 ± 0·03 (6) | — | 87·1 |
| L | 1·25 | 1·16 ± 0·04 (6) | 1·31 ± 0·03 | 86·2 |
| L | 2·5 | 1·29 ± 0·07 (12) | 5·02 ± 1·27 | 88·2 |
| L | 5 | 0·99 ± 0·02 (12) | — | 86·3 |

Each everted sac contained 0·4 ml test solution, and two sacs were immersed in 15 ml of the same solution. Incubation was carried out for 1 hr at 37° under 95% $O_2$/5% $CO_2$. After incubation 0·2 ml aliquots of the mucosal and serosal fluids were added to 4·8 ml 3% aqueous sulphosalicylic acid, protein removed by centrifugation and 1 ml duplicate samples of the supernatant assayed for thiol content with the DTNB reagent, before and after electrolytic reduction. The numbers of sacs are given in parentheses.

* Expressed as the fraction: $\frac{\text{concentration of penicillamine in the serosal fluid}}{\text{concentration of penicillamine in the mucosal fluid}}$

† Standard error of the mean.

TABLE 2. EFFECT OF INHIBITORS ON THE TRANSPORT OF 2·5 mM L-PENICILLAMINE BY EVERTED SACS OF RAT SMALL INTESTINE

| Inhibitor | Concentration ratio of controls Mean ± S.E.M. | Concentration ratio in the presence of inhibitor Mean ± S.E.M. | Statistical significance* | Water content of controls (g/100 g fat-free fresh tissue) Mean | Water content in the presence of inhibitor (g/100 g fat-free fresh tissue) Mean |
|---|---|---|---|---|---|
| 2·5 mM L-methionine† | 1·22 ± 0·05 (6) | 0·94 ± 0·01 (6) | $P < 0·01$ | 87·7 | 87·4 |
| 2·5 mM L-valine | 1·54 ± 0·08 (6) | 1·07 ± 0·03 (6) | $P < 0·002$ | 88·4 | 89·2 |
| 1 mM KCN | 1·36 ± 0·05 (6) | 0·99 ± 0·01 (5) | $P < 0·01$ | 87·7 | 88·1 |
| 2·5 mM L-alanine | 1·52 ± 0·08 (6) | 1·30 ± 0·06 (6) | $P < 0·05$ | 88·3 | 88·6 |
| buffer pH 6·85 | 1·19 ± 0·10 (6) | 0·95 ± 0·04 (6) | $P < 0·02$ | 87·1 | 86·3 |

Four successive sacs were taken from each animal, two outside sacs serving as controls. Total numbers of sacs are in parentheses. Experimental conditions and analytical procedure were as described in Table 1.

* Calculated by applying Student's "*t*"-test to the mean of the difference in concentration ratios between six pairs of sacs. In the case of L-methionine the "*t*"-test was applied to the difference of two means.

† Only in the case of L-methionine were the control sacs taken from the intestine of different animals.

histological examination. The recovery of L-penicillamine in the medium ranged from 80 to 95 per cent, while that of D-penicillamine was mostly 98–100 per cent.

It is evident from Table 1 that L-penicillamine is transported against a concentration gradient across the rat small intestine *in vitro*, whilst under similar conditions D-penicillamine is not transported. The mechanism of this transport of L-penicillamine was energy-dependent, as indicated by the inhibition of transport by KCN (Table 2).

TABLE 3. EFFECT OF PRELIMINARY PYRIDOXINE INJECTION ON THE TRANSPORT OF 2·5 mM L-PENICILLAMINE BY EVERTED SACS OF RAT SMALL INTESTINE

| Concentration ratio in injected rats (Mean ± S.E.M.) | Concentration ratio in controls (Mean ± S.E.M.) | Statistical significance | Water content in sacs from injected animals (g/100 g fat-free fresh tissue) Mean | Water content in control sacs (g/100 g fat-free fresh tissue) Mean |
|---|---|---|---|---|
| 1·19 ± 0·02 (6) | 1·21 ± 0·03 (6) | not significant | 88·1 | 89·3 |

Three hours before the experiment the animals were injected intraperitoneally with 1 ml of a 0·3% (w/v) solution of pyridoxine hydrochloride in 0·9% (w/v) NaCl. Controls were not injected. Two sacs were taken from each animal, starting from proximal jejunum. Total numbers of sacs are in parentheses. Experimental conditions and analytical procedure were as described in Table 1.

TABLE 4. EFFECT OF D- AND L-PENICILLAMINE ON THE TRANSPORT OF L-CYSTEINE

| Inhibitor | Rate of transference in mμmoles/hr per g wet wt. of tissue, mean | | Mean difference of pairs ± S.E.M. | Statistical significance |
|---|---|---|---|---|
| | Control | Inhibitor present | | |
| 2·5 mM D-penicillamine | 55·1 (11) | 80·8 (11) | +25·7 ± 11·1 | $P < 0.05$ |
| 2·5 mM L-penicillamine | 67·3 (12) | 52·3 (12) | −15·0 ± 8·6 | $P < 0.20$ |

Test solutions consisted of 0·1 mM L-cysteine carrier and $^{35}$S-L-cysteine marker, the initial activity of solutions being about 200 counts/sec/0·1 ml. Immediately before the experiment the test solution was made 2 mMolar with respect to dithiothreitol. Experimental conditions were as described in Table 1. Four sacs were taken from each animal, two outside sacs serving as controls. At the end of the incubation period, mucosal fluids and serosal fluids and standards were immersed in boiling water for 10 min and after removal of protein by centrifugation, 0·1 aliquots of the supernatant were taken for radiochemical assay. Conditions of assay are described in the text. Total numbers of sacs are in parentheses.

The capacity of the transport system was very low (Table 1). The active transport was either considerably inhibited, or completely abolished by equimolar L-methionine or L-valine, as well as by a small decrease in pH below the physiological value (Table 2). The effect of equimolar L-alanine on the transport of L-penicillamine was not very pronounced (Table 2). The transport rates were not affected by injecting the animals intraperitoneally with pyridoxine prior to the experiment (Table 3). With the analytical procedure used for estimating penicillamine it was not possible to study the effect of L-cysteine on penicillamine transport. We studied the converse effect, that of D- and L-penicillamine on the transport of 0·1 mM L cysteine containing $^{35}$S-L-cysteine. Penicillamine was used in these experiments at an optimum concentration (2·5 mM) for transport. Dithiothreitol was present in solution to prevent the formation of the mixed disulphide. The results (Table 4) indicated that penicillamine, under the conditions used, altered the transport of L-cysteine. D-isomer stimulated transport, while L-isomer had a slight inhibiting effect.

In tissue uptake studies it was shown (Table 5) that with an incubation period of 4 min the L-isomer was taken up more rapidly than the D-isomer (i.e. that their initial velocities of uptake are different). It was also shown (Table 6) that the uptake of the

TABLE 5. RATES OF UPTAKE OF PENICILLAMINE ISOMERS BY SEGMENTS OF RAT SMALL INTESTINE

| Concentration of isomer | Uptake (μmoles/g fat-free dry wt./4 min) |
|---|---|
| 1 mM D-penicillamine | 4·62 |
| 10 mM D-penicillamine | 31·53 |
| 1 mM L-penicillamine | 6·27 |
| 10 mM L-penicillamine | 58·56 |

Pooled segments from the entire small intestine of two rats were used. Results were corrected for the release of thiol groups into the medium by segments incubated in Krebs phosphate saline at pH 7·4. Analytical procedure is described in the text.

TABLE 6. RATES OF UPTAKE OF L-PENICILLAMINE BY SEGMENTS OF RAT SMALL INTESTINE

| Concentration mM | Uptake (μmoles/g fat-free dry wt./4 min) |
|---|---|
| 1 | 2·88 |
| 2·5 | 6·82 |
| 5 | 16·05 |
| 10 | 27·88 |

Pooled segments from the entire small intestine of two rats were used. Results were corrected for the release of thiol groups into the medium by segments incubated in Krebs phosphate saline at pH 7·4. Analytical procedure is described in the text.

TABLE 7. WATER TRANSPORT BY THE EVERTED SAC OF RAT SMALL INTESTINE

| Added amino acid | Water transport (ml/g fat-free dry wt./hr) Mean | Water content of tissue (g/100 g fat-free fresh tissue) Mean |
|---|---|---|
| None | 3·7 (4) | 88·6 |
| 2·5 mM L-penicillamine | 3·3 (2) | 87·8 |
| 5 mM L-penicillamine | 1·9 (2) | 88·7 |
| 2·5 mM D-penicillamine | 3·0 (2) | 87·9 |
| 5 mM D-penicillamine | 3·3 (2) | 88·9 |
| 2·5 mM L-valine | 3·2 (2) | 87·7 |
| 5 mM L-valine | 3·3 (2) | 88·5 |

Test solutions consisted of 6% (w/v) inulin in Krebs phosphate saline (pH 7·4) containing 0·3% glucose, with or without amino acids at indicated concentrations. Eight sacs were taken from each animal starting from proximal jejunum, and were filled with 0·4 ml of inulin-saline alone, or inulin-saline containing L-penicillamine, D-penicillamine or L-valine, repeating that order twice along the gut. Water transport was calculated from the reduction in inulin concentration in the serosal fluid. Numbers of sacs are given in parentheses.

L-isomer at 1 mM, 2·5 mM, 5 mM and 10 mM shows little evidence of saturation with increasing concentration. Under such conditions, it is impossible to decide whether the uptake is a combination of a saturatable with a non-saturatable process, or whether it is simply a high-$K_m$ saturatable process.[36]

The effect of both penicillamine isomers on the transport of water by the everted intestinal sacs was of the same order of magnitude as that reported to occur in the presence of other amino acids[37] (Table 7).

## DISCUSSION

The inhibition of the active transport of L-penicillamine by L-valine and L-methionine indicates that L-penicillamine shares the transport pathway of the neutral amino acids. The almost complete abolition of L-penicillamine transport by equimolar L-valine indicates a considerable disparity in the transport constants between L-penicillamine and L-valine, i.e. a much higher $K_m$ for L-penicillamine.

The failure to demonstrate the active transport of L-penicillamine with everted sacs at a higher concentration indicates that 5 mM L-penicillamine already inhibits its own transport against a concentration gradient. This is especially evident since the uptake of L-penicillamine by tissue segments proceeds even at 10 mM with but small evidence for saturation.

The $pK$ value of the thiol group in penicillamine is 8·1 which means that at pH 7·5 the thiol group is 7% ionized.[38] It is highly probable that this group also may be involved in transport, possibly in a non-specific manner. The stimulation of the transport of L-cysteine by D-penicillamine may be the result of its effect on exchange diffusion, indicating the involvement of penicillamine in the carrier system of L-cysteine. The discrepancy in the effect of two penicillamine isomers on the transport of L-cysteine may possibly be a result of their different intracellular levels. Also, the effect of D-penicillamine may be non-specific whilst that of L-penicillamine may be a result of a compensation between a non-specific action and a substrate-specific carrier-binding.

Great individual variations found in the rates of transport of L-penicillamine by the everted sacs may also be a reflection of the manifold involvement of this compound in the metabolism of the cell, such as reaction with PLP, either free or protein-bound, its metal-chelating properties, participation in disulphide exchange, etc. A comparative study of the incorporation of the tritium-labelled D- and L-penicillamine in the liver and kidney of rats has shown that the L-isomer is incorporated to a much greater extent in these tissues.[39] Tissue binding might be a reason for our finding that the transport rates at 1·25 mM L-penicillamine are less than one half of the rates shown for 2·5 mM L-penicillamine (Table 1).

Amino acid transport across the intestinal mucosa is known to be PLP-dependent.[40–42] The ability of penicillamine to combine with PLP does not seem, however, to affect its own transport. This is indicated by the fact that transport rates were not affected by preliminary injection of the animal with pyridoxine.

Certain pharmacologically-active amino acids are actively transported across the cell membrane to the site of action, e.g. the carcinostatic 1-amino-cyclopentane-1-carboxylic acid.[2] The toxic amino acid analogue L-penicillamine has now been shown to also belong to this category.

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