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TRANSPORT OF D- AND L-PENICILLAMINE BY MAMMALIAN CELLS

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SUMMARY

L-Penicillamine is taken up and accumulated by mammalian cells via their amino acid transport systems. Natural amino acids compete with L-penicillamine and have a higher affinity to their transport systems, as shown in the case of L-valine.
D-Penicillamine is not accumulated within the cells and does not compete with natural amino acids.

INTRODUCTION

D-Penicillamine (DPA)[1], an amino acid derived from penicillin, is therapeutically used in treatment of Wilson's disease, cystinuria, rheumatoid arthritis, poisoning by heavy metals, and various other diseases (for review see 1, 2). Side effects of this drug, which is toxic in animals (3) and microorganisms (4), are mainly referred to chelating of trace metals and a pyridoxal phosphate (vitamin B_6) antagonism. In addition, small amounts of L-penicillamine (LPA)[1] contaminating commercial products are suggested to be the reason of untoward reactions, since it is known that LPA can be incorporated in proteins of bacteria (5) and mammalian cells (6, 7) in competition with L-valine. However, the affinity of L-valine to its cognate tRNA is at least 500 times higher than that of LPA (7) and the content of contaminating LPA in commercial DPA of today is as low as 0,1% or less (8). Therefore, on compar-

[1] Abbreviations: DPA - D-penicillamine; LPA - L-penicillamine

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ing the possible serum concentrations (about 3 μM[1]) for LPA and 168 - 317 μM for L-valine), a considerable incorporation of LPA in the proteins of patients treated with DPA is rather unlikely, provided that an accumulation of LPA within the cells by means of the amino acid transport system has not to be taken into account. To answer this question the few data available concerning the mechanism of uptake of penicillamine by mammalian organisms are not sufficient: Wass and Evered (8) investigated the transport of penicillamine into everted sacs of small intestine from rat and found a moderate accumulation of LPA by an active transport through the intestinal mucosa inhibited by L-methionine and L-valine. DPA was found not to be transported against the concentration gradient, though it was taken up by the segments of small intestine to a lesser extent.Experiments on the distribution and excretion of ^{14}C-labeled penicillamine, performed by Planas-Bohne (10) also indicated a more effective uptake and retention of LPA in the different organs of rats as compared to DPA. From plasma clearance the extra- and the intracellular water were calculated to be the physiological distribution spaces of both penicillamine enantiomers. Compartmental studies led to the conclusion that the extracellular fluid is the primary distribution volume of both DPA and LPA (11).

In this paper, experiments on the uptake of DPA and LPA by some mammalian cell lines are described. They led to the conclusion that there is no accumulation of DPA at all and, as compared to L-valine, a slower accumulation of LPA within mouse fibroblasts.

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[1] calculated from oversimplified conditions: a rather high, single dose of 3,6 g DPA containing at most 0,1% LPA, 65% resorption and the blood volume as distribution space only.

MATERIALS AND METHODS

[^{14}C]-L- and D-penicillamines (specific activity 1 respectively 1,4 mCi/mMol) synthesized by Gesellschaft für Kernforschung, Karlsruhe, were kindly supplied by Heyl & Co., Berlin, unlabeled penicillamines were gifts of Heyl & Co., Berlin, and Chemiewerk Homburg, Frankfurt/M.or were purchased from Serva, Heidelberg. Tritium-labeled amino acids were obtained from NEN Chemicals GmbH, Dreieichenhain.

Cells: L 929 mouse fibroblasts were purchased from Flow laboratories GmbH, Köln; A 9 mouse fibroblasts were kindly supplied by Institut für Genetik, Köln. Human amnion epithelial cells were prepared and kindly supplied as were human embryonic fibroblasts and human prostate epithelial cells by Dr. M. Geissler, Institut für Humangenetik der Universität Frankfurt.

Media: For cultivation of cells minimal essential medium (MEM) with Hank's salts and 10% newborn calf serum was used. Amino acid uptake experiments were performed with Earle's salt solution containing 0,5% glucose.

Uptake of [^{14}C]D- and [^{14}C]L-penicillamine by mammalian cells: A 9 cells were cultivated in Petri dishes of 3 cm diameter until nearly confluent monolayers had formed (about $1 \cdot 10^6$ cells). The monolayers were washed with Earle's solution containing 0,5% of glucose. 1,5 ml of prewarmed mixtures of ^{14}C-labeled penicillamine (0,33 mM) and Earle's solution were added, and the cultures were then incubated at 37°C for 2, 5, 10, and 20 min, respectively The incubation was stopped, washing the cultures with Earle's solution (37°C) and treating with 1 ml of a 0,5% trypsin solution for 15 min were the next steps. The cell suspensions were transferred to scintillation vials and evaporated to dryness. The residues were dissolved in 0,5 ml of a tissue solubilizer and the radioactivity was determined using a toluene-based scintillator.

Competition of natural amino acids and penicillamine during uptake by mammalian cells: Cells were cultivated in Petri dishes of 6 cm diameter until nearly confluent monolayers had formed (about $5 \cdot 10^6$ cells). The monolayers were washed with Earle's solution. 1,5 ml of prewarmed mixtures of penicillamine and amino acid (final concentration 33 µM and 3,3 mM, respectively) in Earle's solution were added, and the cultures were incubated for 2 min at 37°C. Then the incubation was stopped by washing the cultures (Earle's

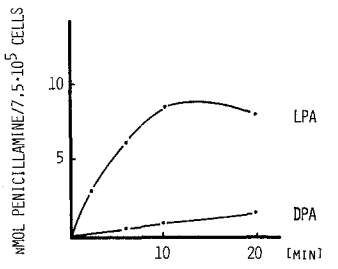

FIGURE 1 UPTAKE OF [^{14}C]D- AND [^{14}C]L-PENICILLAMINE BY MOUSE FIBROBLASTS

A 9 Cell monolayers were cultivated in Petri dishes of 3 cm diameter. Earle's solution was used as medium during amino acid uptake. Concentration of penicillamine was 0,33 mM. The points given in the figure are mean values resulting from triplicate experiments.

solution, 37°C). The monolayers were treated with 1,5 ml of a 0,5% trypsin solution for 15 minutes. The cell suspensions thus obtained were added to 10 ml of a dioxane-based liquid scintillation-cocktail and counted for radioactivity.

Determination of cell volume: A small volume of a concentrated cell suspension of A 9 cells (about $4 \cdot 10^6$ cells/ml) was mixed with tritiated water and [^{14}C]dextran (60 - 90 000 D). After a short time during which tritiated water equilibrated between extra- and intracellular water and dextran remained in the extracellular fluid, aliquots of this suspension (100 µl) were rapidly centrifuged (152 Microfuge-Beckman) in small polyethylene tubes through a layer (70 µl) of silicon oil (AR 20, Wacker-Chemie, München) into 30 µl of 12% perchloric acid. The aqueous contents of the tubes were frozen, the tips of the tubes were removed by a razor and the radioactivity of the perchloric acid layer was then determined by means of a toluene/ethylene glycol monomethyl ether-based scintillator. The cell volume was calculated from the carbon and tritium counts per milliliter of the cell suspension prior to centrifugation and the counts of the perchloric acid layer after centrifugation, carbon counts now representing external water adsorbed to the cells, tritium counts representing internal cellular water plus external water.

RESULTS AND DISCUSSION

In the absence of other amino acids LPA is easily taken up by mouse fibroblasts as can be seen from Figure 1. From incubation

TABLE 1 ACCUMULATION OF [^{14}C]L- AND [^{14}C]D-PENICILLAMINE BY MOUSE FIBROBLASTS

A 9 Cell monolayers were cultivated in Petri dishes of 3 cm diameter. Earle's solution was used as medium during amino acid uptake. Concentration of amino acids: 0,33 mM labeled penicillamine (except Nr. 3); 0,33 mM [^{3}H]L-valine.

Exp. Nr.	Amino acid added	Incubation time [min]	cpm/ml in the medium	cpm/ml in the cells	Accumulation factor[1)]
1	[^{14}C]LPA	10	$5,8 \cdot 10^{5}$	$3,94 \cdot 10^{7}$	68
2	[^{14}C]LPA in complete medium	10	$5,8 \cdot 10^{5}$	$5,4 \cdot 10^{7}$	0,9
3	[^{14}C]LPA (2,5%) in unlabeled DPA	10	$0,17 \cdot 10^{5}$	$0,12 \cdot 10^{7}$	73
4	[^{14}C]DPA[2)]	10	$7,3 \cdot 10^{5}$	$0,17 \cdot 10^{7}$	
5	[^{14}C]DPA[3)] - 2,5% LPA	10	$7,1 \cdot 10^{5}$	$4,25 \cdot 10^{5}$	0,6
6	[^{14}C]LPA	3	$5,8 \cdot 10^{5}$	$9,3 \cdot 10^{6}$	16
7	[^{3}H]L-valine[4)]	3	$9,9 \cdot 10^{4}$	$6,8 \cdot 10^{6}$	69

1) Accumulation factor: cpm/ml in the cells divided by cpm/ml in the medium

2) containing a contamination of 2,5% LPA

3) counts from experiment Nr.4 after subtraction of counts from experiment Nr.3

4) same concentration of L-valine as LPA concentration in experiment Nr.6

times exceeding 10 min the radioactivity taken up by the cell cultures decreased. This may be a result of cell death caused by suboptimal cultivation conditions in Earle's solution and by decrease of LPA concentration due to oxidation forming disulfide. Therefore, in the following experiments incubation times were reduced as far as possible.

In the case of DPA, it had to be considered that the ^{14}C-labeled DPA preparation used in these experiments contained a contaminat-

ion of 2,5% of the L-isomer (8), which is taken up preferentially. Thus, only a very small amount of DPA really seems to be taken up by the cells.

To confirm these results, experiments were performed, which allowed to calculate the accumulation of amino acids in the intracellular fluid. For this purpose, the cell volume of A 9 cells used in these experiments was determined following the method of Pfaff and Klingenberg (12), modified for cell volume determinations by Lenssen (13). It was found to be 1880 $(\mu m)^3$.

The results of these experiments and calculations are given in Table 1. It is obvious that LPA is concentrated within the cells while DPA is not. The results from experiment Nr.5, corrected for LPA content of [^{14}C]DPA, suggests the cellular DPA concentration to remain lower than the extracellular concentration.

Since there was no doubt about it that the accumulation of LPA is performed by the normal amino acid transport system of the cell, it was of great interest to see whether there is a specific competition between LPA and one or another of the natural amino acids. Such a competition was pointed out earlier in the case of LPA aminoacylation, which was specifically inhibited by L-valine (7).

From Table 2 it can be seen that LPA competes more or less with all the ten amino acids tested for its uptake by mouse fibroblasts. The specificity of this competition was much lower than observed in bacteria (unpublished results), a result, which is in aggreement with the lower specificity of the mammalian amino acid transport system as compared to that of bacteria. In the contrary, DPA does not influence the uptake of any of the 10 amino acids. It has to be pointed out that the results for amino acid uptake are taken from the approximately linear area of the time-uptake curves.

TABLE 2 INFLUENCE OF D- AND L-PENICILLAMINE ON THE UPTAKE OF VARIOUS AMINO ACIDS BY MOUSE FIBROBLASTS

Monolayers of mouse fibroblasts (A 9 and L 929) were cultivated in Petri dishes of 6 cm diameter. Earle's solution was used as medium during amino acid uptake. Concentration of amino acids: 33 µM tritium-labeled amino acid; 3,3 mM LPA and DPA,respectively. Incubation time: 2 minutes. Results are given in % of control (uptake of amino acids by the cells in the absence of penicillamine). They are mean values of 2 - 10 separate experiments with 3 Petri dishes each.

Amino acid	Relative uptake of amino acids by mouse fibroblasts in the presence of	
	LPA	DPA
L-Valine	34	98
L-Leucine	27	97
L-Alanine	69	100
L-Threonine	46	95
L-Phenylalanine	50	100
L-Methionine	43	97
L-Proline	80	94
L-Arginine	87	100
L-Histidine	31	100
L-Glutamic acid	74	95

One of the most effective competitive inhibitors of LPA uptake by mouse fibroblasts is L-valine. Therefore, L-valine was used to compare the uptake of LPA with that of a natural amino acid. (Table 1, experiment Nr.6 and 7). The result was that within 3 min L-valine showed an accumulation four times higher than that of LPA, thus reflecting a higher affinity of L-valine to the transport system.

The competition between LPA and L-valine was also used to demonstrate that there is no difference in DPA as well as LPA uptake in several mouse respectively human cell lines (Table 3). Owing

TABLE 3 INFLUENCE OF D- AND L-PENICILLAMINE ON THE UPTAKE OF [^{3}H]L-VALINE BY SEVERAL MAMMALIAN CELL LINES[1)]

Cell line	Relative uptake of [^{3}H]L-valine in the presence of	
	LPA	DPA
Mouse fibroblasts		
L 929 cells	32	96
A 9 cells	32	100
Human cells		
Amnion cells	34	100
Embryonic cells	43	94
Prostate cells	31	95

1) For legend see Table 2

to the low availability of labeled DPA and LPA, these experiments were carried out with labeled amino acids to be taken up and unlabeled penicillamine as inhibitor.

It can be summarized that LPA - in contrast to DPA - is actively transported into mammalian cells by the transport system meant to ensure cellular amino acid supply. The affinity of the transport system is lower to LPA than it is to the natural amino acid L-valine. Therefore, under physiological conditions, as approximated by experiment Nr.2 (Table 1), at normal levels of L-valine and other amino acids no accumulation of LPA within the cells can be expected. Thus, the question can be denied whether or not small amounts of LPA contaminating DPA, used therapeutically, may be fatally concentrated by the patients' cellular transport systems.

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