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# Metabolism of pyrimidine L-nucleosides

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## ABSTRACT

The intraperitoneal application of L-nucleosides (L-Cyd, L-Urd, L-dThd) to mice results in distribution of these compounds into tissues of the organism and their gradual excretion in the unchanged form. The residual level has been observed with L-ribonucleosides only and contains in addition to the L-nucleoside its 5′-phosphate. The phosphorylation in vivo is catalyzed by nucleoside-kinase and utilizes ATP as the phosphate donor while glycerol 1-phosphate and creatine phosphate are inactive. The L-cytidine derivatives are in vivo deaminated to the derivatives of L-uridine. On the other hand, when L-uridine is applied in vivo, derivatives of L-cytidine are obtained on the level of both the nucleoside and 5′-ribonucleotide.

## INTRODUCTION

L-Nucleosides are enantiomeric derivatives of naturally occurring nucleic acid components and have not been so far found in Nature. Their chemical properties are identical with those of D-enantiomers as regards the steric arrangement of the molecule, distribution of electrons and the related effects such as activity of particular groups, conformation and the like. However, when some step of the reaction comprises interaction of the nucleoside with a chiral molecule, e.g., with another nucleoside or nucleotide[1] or an enzyme molecule (in enzymatically catalyzed reactions), it may be inferred from affinity comparison of D- and L-nucleosides or their derivatives whether the interaction includes the chiral portion of the substrate molecule.[2,3]

As shown in earlier reports from this Laboratory, the L-nucleosides neither penetrate the bacterial cells of E.coli and B.subtilis[4] nor interfere with the penetration of natural-

ly occurring nucleosides through the bacterial cell membrane.[5,6] On the other hand, L-adenosine when applied intraperitoneally to mice is partly excreted in the unchanged form and partly retained in various tissues of the organism. This retention is accompanied, especially in liver cells, by phosphorylation with the formation of L-AMP (cf.[7]). In the present paper we wish to report investigations on the metabolism of pyrimidine L-nucleosides with the use of the same model, namely, the intraperitoneal application to mice.

## EXPERIMENTAL

### Materials

L-Uridine and L-cytidine were prepared according to ref.[8]. L-Uridine-5-$^{3}H$ and L-cytidine (spec.act. 0.2 Ci/mmol) were obtained by tritiation of the corresponding 5-bromo derivatives.[9] 2′-Deoxy-L-thymidine was synthesized according to ref.[10] 2′-Deoxy-L-thymidine-5-$CH_3$-$^{14}C$ (0.047 Ci/mmol) was obtained according to ref.[11] ATP ($Na_2$ salt), creatine phosphate (Na salt), and glycerol 1-phosphate (Na salt) were preparations of Calbiochem (Los Angeles, USA). The snake venom (Crotalus adamanteus) 5′-nucleotidase was the preparation of Worthington, USA.

### Excretion and Distribution of L-Nucleosides in Tissues

Excretion dynamics of L-nucleosides by urine and distribution of L-nucleosides in tissues was examined with the use of "H" mice. L-Nucleosides were applied in the following concentrations: L-Cyd-$^{3}H$, 2 $\mu$Ci (4.0 $\mu$g) per mouse; L-Urd-$^{3}H$, 2 $\mu$Ci (2.4 $\mu$g) per mouse, and L-dThd-$^{14}C$, 1 $\mu$Ci (4.8 $\mu$g) per mouse. Standard food was administered (water ad lib.). Urine was collected in indicated intervals with the use of Whatman No. 3 MM filter paper, the paper eluted, and aliquots (0.05 ml) measured in the dioxane scintillation solution. Tissue aliquots (10 mg) were homogenized in 70% aqueous ethanol acidified previously with acetic acid and the radioactivity measured in an 1:1 mixture of the dioxane and the toluene scintillation solution.

Phosphorylation of L-nucleosides in vivo. L-Nucleosides were applied in doses 10 $\mu$Ci per mouse (L-cytidine and L-uridine) and 5 $\mu$Ci per mouse (L-dThd). Animals were killed in indicated

intervals and aliquots of investigated tissues homogenized in cold 1% TCA (100 mg/2 ml). The concentration of TCA was then adjusted to 10%, the mixture kept in ice for 30 min, and centrifuged. The supernatant was successively extracted with two portions of ether and then with ethanol-ether (3:1). The sample was adsorbed for 30 min to active charcoal in weakly acidic medium and the coal desorbed with a mixture of 25% aqueous methanol and ammonia (30 : 0.5, v/v). The sample was then chromatographed on Whatman paper No. 3 MM in the solvent system isobutyric acid-water-aqueous ammonia (66 : 33 : 1.5). Bands of nucleosides and nucleotides were eluted with water and the radioactivity of eluates measured in the dioxane scintillation solution.

Analysis of the nucleotide fraction. An aliquot of the nucleotide band residue (see above) (5 000 c.p.m.) was incubated in 0.05 M TRIS-HCl (pH 9) (100 µl) with the snake venom (Crotalus adamanteus) 5′-nucleotidase (20 µg) at 37°C for 3 h and the mixture chromatographed on Whatman No. 3 MM paper in the solvent system 2-propanol-concentrated aqueous ammonia-water (7 : 1 : 2) using the corresponding nucleoside and nucleotide as markers. The appropriate bands were eluted with water and the radioactivity of eluates measured in the dioxane scintillation solution.

Analysis of the nucleoside fraction. An aliquot of the nucleoside band residue (10 000 c.p.m.) was analyzed by electrophoresis on the Whatman No. 3 MM paper in 0.1 M triethylammonium borate (pH 7.5) (40 V/cm, 1 hour) using the corresponding nucleoside and base as markers. Bands of the base and the nucleoside were eluted with water and the radioactivity of the eluate measured in the dioxane scintillation solution.

Crude enzymatic preparation from homogenized mouse liver.

Mouse liver tissue was homogenized in 0.012 M TRIS-HCl buffer solution (pH 7.5) (ratio, 1:5, w/v) at 0°C, subjected to sonication in 6 x 10 sec intervals, and centrifuged at 39 000 r.p.m. at 4°C for 2 h. The supernatant was dialyzed against 1 mM TRIS-HCl buffer solution (pH 7.5) in a cooled room for 24 h (three replacements of the dialysis media). Proteins in the supernatant were determined by the method of

Lowry.[12]

Phosphorylation of D-cytidine and L-cytidine in the presence of homogenized rat liver

A) Influence of the presence of ATP. - The incubation mixture (250 µl) contained 0.1 M TRIS-HCl (pH 7.5), 4 mM $MgSO_4$, 0.5 mM Cyd (0.043 µCi of D-Cyd-$^{14}$C) or L-Cyd (1.0 µCi of L-Cyd-$^{3}$H), the dialyzed liver homogeneate (150 µg), and ATP ($Na_2$ salt) in stated amounts. The mixture was incubated at 37°C for 30 min, chromatographed in the solvent system 2-propanol-concentrated aqueous ammonia-water (7 : : 1 : 2). For the results see Fig. 6.

B) Influence of the concentration of rat liver homogeneate. - The incubation mixture (250 µl) contained 0.1 M TRIS-HCl (pH 7.5), 4 mM $MgSO_4$, 0.2 mM ATP, 0.5 mM D-Cyd (0.043 µCi of D-Cyd-$^{14}$C) or L-Cyd (1.0 µCi L-Cyd-$^{3}$H) and the stated amount of the dialyzed liver homogeneate. The mixture was incubated at 37°C for 30 min and chromatographed analogously to paragraph A. For the results see Fig. 5.

C) Influence of the phosphate donor.- The incubation mixture (250 µl) contained 0.2 mM TRIS-HCl (pH 7.5), 4 mM $MgSO_4$, 250 µg of the dialyzed liver homogeneate protein, 0.5 mM D-Cyd (0.043 µCi of D-Cyd-$^{14}$C) or L-Cyd (1.0 µCi of L-Cyd-$^{3}$H), and 0.25 mM ATP or 0.3 mM glycerol 1-phosphate or 0.15 mM creatine phosphate. The mixture was incubated at 37°C for 45 min, diluted with an equal volume of 99% ethanol, kept at 0°C for 1 h, centrifuged, and the supernatant chromatographed analogously to paragraph A. For the results see Table II.

## RESULTS AND DISCUSSION

In investigations on the biological function of L-enantiomers of the naturally occurring nucleosides in organism, it was of interest to examine their distribution in particular organs, level dynamics of active products in blood, changes of applied substances, and finally, their excretion from the organism. The intraperitoneal application of L-nucleosides to mice results in a rapid and massive excretion in a metabolically unchanged form in the early minutes after the adminis-

tration (Fig.1). The course of the excretion in urine is almost linear up to the fourth hour after the application (more than 80% of the applied dose is excreted in this period of time). Excretion then remains almost constant up to the 24th hour. As determined by analysis of urine, all the radioactivity was excreted in the form of the nucleoside and not as a heterocyclic base.

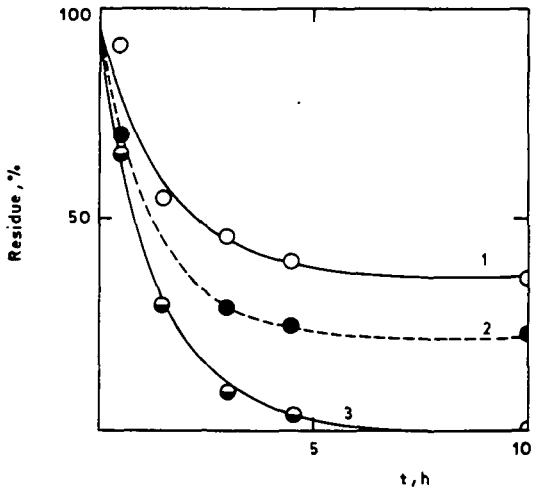



Fig.1 L-Nucleoside excretion by mouse urine. 1, L-Cyd; 2, L-Urd; 3, L-dThd

A certain amount of residual activity remaining in the organism (especially after the application of L-cytidine) indicates metabolic transformations of the applied L-nucleosides in vivo. As confirmed by distribution of the radioactivity in various tissues of the organism (Figs 2 and 3), the L-nucleosides do not selectively pass from blood into some particular tissues. The distribution pattern of all the three applied L-nucleosides is very similar. The observed highest radioactivity level in kidney corresponds to the obviously specific function of kidney in excretion of L-nucleosides.

Similarly to L-adenosine[7], the residual activity of L-nucleosides of the pyrimidine series in cells of particular tissues is proportional to the extent of phosphorylation in cells as it may be inferred from data of Fig.4 and Table I, whereas L-cytidine and L-uridine are in vivo phosphorylated

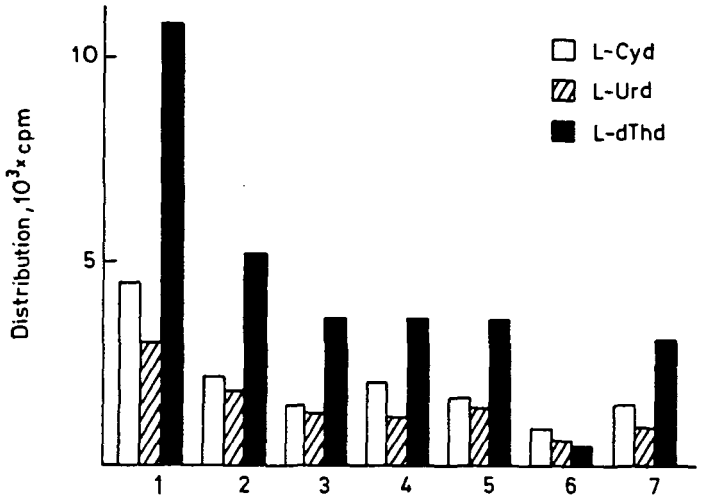



Fig.2 Distribution of radioactivity in various tissues (extrapolated for $t_0$). 1, kidney; 2, liver; 3, thymus; 4, spleen; 5, heart; 6, brain; 7, muscle

especially in metabolically active organs (liver, kidney), the phosphorylation of 2′-deoxy-L-thymidine occurs to a minimum extent only in these and other investigated organs. Since the reverse penetration of L-nucleotides through the cell wall appears to be more difficult than in the case of the corresponding L-nucleosides and because of the obviously low level of the unspecific phosphomonoesterase (capable of dephosphorylating the L-nucleotides[2]), the L-nucleotides are cumulated in cells.

Phosphorylation of L-nucleosides affords the 5′-nucleotide derivatives as it has been earlier determined on the phosphorylation of L-adenosine in vivo in liver cells[7] and confirmed in the present work also for the case of L-uridine, L-cytidine and 2′-deoxy-L-thymidine by analysis of the radioactive nucleotide portion in the homogeneate of liver cells after the application of these L-nucleosides. This fraction (obtained by paper chromatography) is resistant towards the action of the snake venom 5′-nucleotidase capable of degrading exclusively the D-5′-nucleotides[2,7] and not their L-enantiomers. Consequently, the fraction contains a L-5′-nucleotide.

The character of the phosphorylation reaction of L-nucleo-

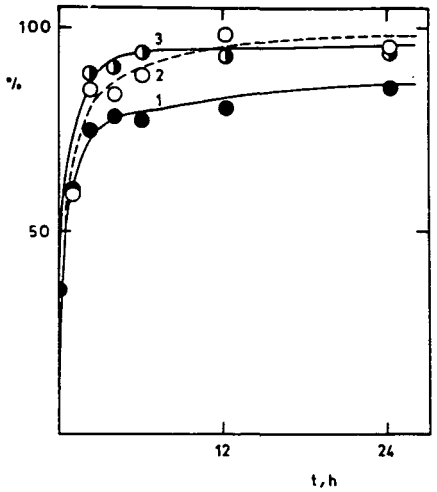



Fig.3 Time dependence of L-nucleoside distribution in various tissues. 1, L-Cyd; 2, L-Urd; 3, L-dThd

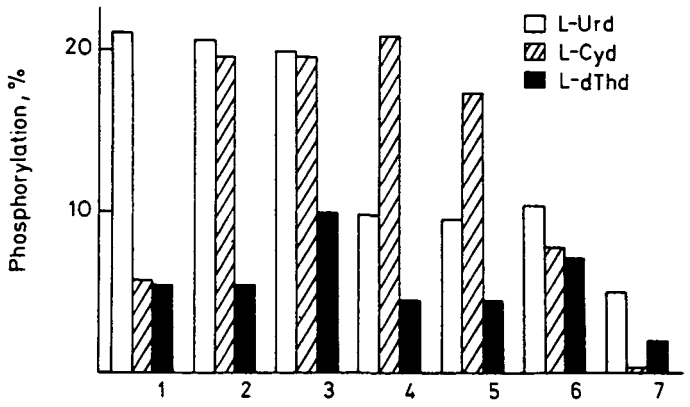



Fig.4 Phosphorylation of L-nucleosides in various tissues. 1, blood; 2, kidney; 3, liver; 4, thymus; 5, spleen; 6, heart; 7, muscle

sides was investigated in vitro systems containing purified supernatant of the mouse tissue homogeneate from which the internal inorganic phosphate was removed by dialysis. As the model substrate of the phosphorylation reaction, L-cytidine was selected and its behaviour compared with that of D-cytidine (i.e., naturally occurring cytidine). ATP, creatine phosphate, and glycerol monophosphate were used as the source of phosphoric acid (the latter two substances as substrates for

TABLE I Metabolism of L-Nucleosides in vivo (data in %)

| | Blood | Kidney | Liver | Thymus | Spleen | Heart | Muscle |
|---|---|---|---|---|---|---|---|
| | | L-Uridine | | | | | |
| Phosphorylation | 22.0 | 21.0 | 19.5 | 9.5 | 9.0 | 10.5 | 5.0 |
| Amination[a] | 27.7 | 43.7 | 52.1 | 20.9 | 20.2 | 42.0 | 21.0 |
| L-CMP[b] | 68.0 | 37.0 | 29.0 | 30.0 | 50.0 | 30.0 | 31.0 |
| L-UMP[c] | 9.0 | 13.0 | 13.0 | 5.0 | 0 | 0 | 0 |
| | | L-Cytidine | | | | | |
| Phosphorylation | 6.5 | 19.0 | 19.0 | 21.5 | 17.0 | 8.0 | 0 |
| Deamination[d] | 32.0 | 38.9 | 50.7 | 17.0 | 22.8 | 28.4 | 30.2 |
| CMP-Deamination | 41.0 | 14.0 | 24.0 | 0 | 0 | 0 | 0 |
| Cyd-Deamination | 23.5 | 26.0 | 34.5 | 16.0 | 23.0 | 22.0 | 25.0 |
| | | 2′-Deoxy-L-thymidine | | | | | |
| Phosphorylation | 5.8 | 6.2 | 10.3 | 4.0 | 3.6 | 7.0 | 2.2 |

[a] Formation of cytosine derivatives corrected with respect to the reverse deamination; [b] referred to total cytosine derivatives; [c] referred to total uracil derivatives; [d] average, 28.2 ± 2.0% .

phosphotransferases). Table II shows results of the phosphorylation of both enantiomeric nucleosides with the use of ATP, creatine phosphate, and glycerol monophosphate or their combinations. From this group, only ATP was the donor while the other two monoesters of phosphoric acid exhibited an inhibitory effect on the course of the phosphorylation reaction, i.e. were not substrates. It may be seen that the phosphorylation depends on the concentration of both the protein homogeneate (Fig.5) and ATP as the phosphate donor (Fig.6), the D-cytidine being in both cases a better substrate than the L-enantiomer. It may be inferred from these observations that enzymes that catalyze the phosphorylation of L-nucleosides to L-5′-nucleotides in the presence of ATP as the phosphate donor, are nucleoside kinases. Since 2′-deoxy-L-thymidine is in the above phosphorylation in vitro (Table I) a considerably worse substrate than L-uridine or L-cytidine, the enzymes appear to be markedly specific with respect to the sugar moiety of the nucleoside.

After the application of L-cytidine in vivo, the cell homogeneate contains not only the expected cytosine derivatives

TABLE II Influence of Various Phosphate Donors upon the Phosphorylation of Cytidine and L-Cytidine with Rat Liver Homogeneate

| ATP | Creatine phosphate | Glycerol 1-phosphate | L-CMP % | D-CMP % |
|---|---|---|---|---|
| - | - | - | 1.5 | 1.2 |
| + | - | - | 20.2 | 30.8 |
| - | + | - | 1.7 | 1.4 |
| - | - | + | 1.2 | 0.8 |
| + | + | - | 14.4 | 20.0 |
| + | - | + | 17.6 | 26.0 |
| - | + | + | 1.6 | 1.8 |
| + | + | + | 7.5 | 12.0 |

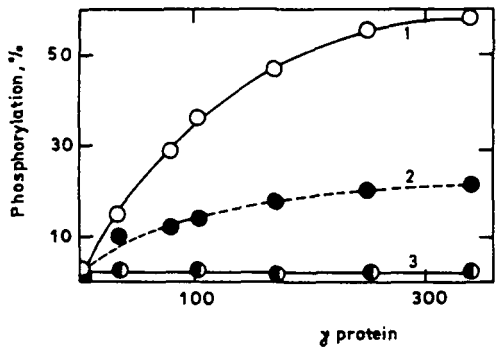



Fig. 5 D- and L-Cytidine phosphorylation in the presence of mouse liver homogenate (ATP as phosphate donor). 1, D-Cyd; 2, L-Cyd; 3, D-Cyd (ATP absent)

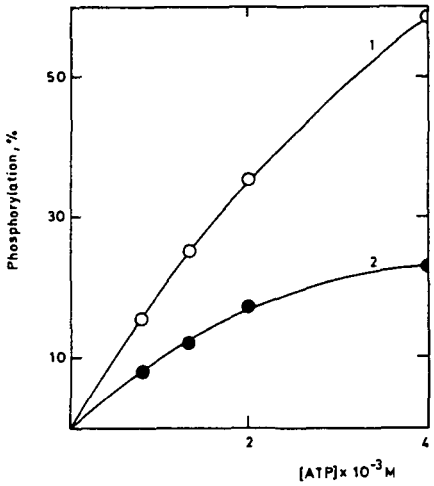



Fig. 6 The dependence of D- and L-cytidine phosphorylation in the presence of mouse liver homogenate upon ATP concentration

(L-Cyd, L-CMP) but also the corresponding uracil derivatives such as L-Urd and L-UMP (Table I). This deamination of cytosine derivatives is a usual reaction also observed in the case of cytosine itself; the deamination does not almost depend on the character of the sugar moiety of cytosine[13,14]. As shown (Fig.7) by dependence of the deamination extent on the degree of the phosphorylation of L-cytidine in vivo in analyzed tissues, this reaction proceeds on the level of both the nucleoside and nucleotide being thus independent on the phosphorylation. L-CMP appears to be a somewhat worse sub-

strate than L-Cyd.

When applied in vivo, L-uridine is mostly converted to L--cytidine derivatives (L-Cyd, L-CMP) (Table I). This transformation may be observed in all the analyzed tissue samples

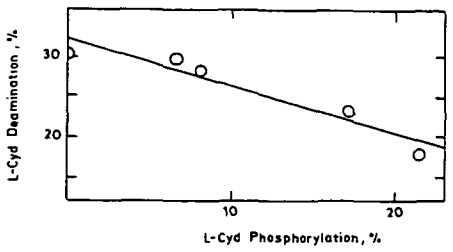



Fig. 7 The dependence of L-cytidine deamination upon its phosphorylation

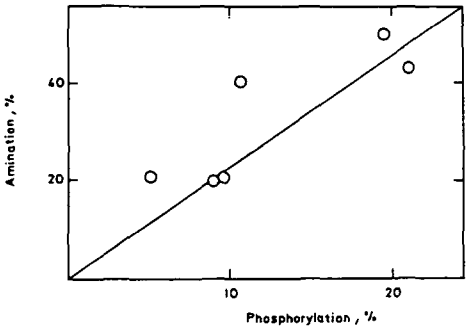
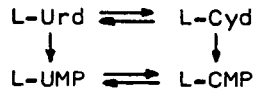



Fig. 8 The dependence of L-Urd L-Cyd conversion upon total nucleoside phosphorylation in various tissues

except for the cerebral tissue in which the overall level of the L-nucleoside is too low. The highest conversion degree has been observed in liver and kidney cells. This transformation to cytosine derivatives ("amination") is accompanied by the above mentioned deamination reaction; the results shown in Table I are therefore corrected with respect to this reverse reaction.

The amination of L-uridine in vivo affords L-cytidine and L-CMP. Since the degree of the reverse dephosphorylation of L-CMP is very low if any (vide supra), L-cytidine is obviously formed directly on the level of a nucleoside. The main portion of the nucleotide portion consists of L-CMP, the enantiomeric purity of which was established by resistance towards the action of the snake venom 5′-nucleotidase (vide supra). As shown (Fig. 8) by dependence of the overall amination of L-uridine on the phosphorylation in analyzed tissues, the conversion is coupled with the phosphorylation. Thus, when L-uridine is applied in vivo, a complex of reactions manifests itself including amination of uracil derivatives and deamination

of cytosine derivatives on the level of both nucleosides and the corresponding 5′-phosphates. L-UMP has been observed to be a better substrate for the amination than L-uridine.




The reaction may be catalyzed in both directions by the same enzyme or related enzymes; it is obviously part of the salvage-pathway apparatus of eukaryotic cells. It may be assumed that this reaction is not limited to L-nucleosides but also takes place in the series of D-ribonucleosides or their derivatives.

The transformation of uracil derivatives to cytosine derivatives has been so far known exclusively on the level of 5′-triphosphates (UTP ⟶ CTP)[14] but not on the level of nucleosides or mononucleotides. The existence of this transformation could not be discovered with the use of the naturally occurring nucleoside, namely, D-uridine since this substance undergoes complex reactions when applied in vivo (e.g., phosphorolysis to uracil). On the other hand, L-uridine and L-cytidine or their 5′-nucleotides are neither phosphorolyzed nor degraded. The present work exemplifies the use of L-nucleotides for identification of biological transformations in complex systems.

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