Transport of nucleosides in *Bacillus subtilis*: characteristics of cytidine-uptake

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ABSTRACT

In *Bacillus subtilis* SMYW cytidine and uridine are transported by a common system. Transport of these substances requires metabolic energy. After 60 sec of ^3H -cytidine-uptake practically all accumulated radioactivity was found in phosphorylated products. Addition of ribonucleosides with inhibitory effect upon cytidine-uptake resulted in competitive type of inhibition while interference with deoxyribonucleosides was of hyperbolic competitive type. Adenosine possesses a high affinity to cytidine-transporting site but requires different system(s) for its own transport. Guanosine, adenine, cytosine and 5²-nucleotides do not interfere with cytidine-uptake.

INTRODUCTION

A common feature of nonelectrolyte-transport is the plurality of transporting systems operating even within the same species (see review by Kotyk¹). This is also valid for the uptake of nucleosides in bacteria, which was so far mostly investigated in *E. coli* and its mutants²⁻¹⁰. This process was found to be active and in most cases mediated by more systems with different specificities.

However, few reports exist on nucleoside-transport in *B. subtilis*, which evolved in habitats with lower concentration of nucleic acid components than Enterobacteriaceae. Beebe¹¹ pointed out the general importance of phosphatidylethanolamine as a membrane component in the membrane transport function because its deficiency in a mutant of *Bacillus subtilis* was accompanied by a decrease of initial uptake rates of different metabolites including uridine and adenosine. Richardson¹², investigating the effect of phenethylalcohol on *B. subtilis* transformation, found reduced ability to transport 2'-deoxythymidine and other compounds.

The present paper deals with general characteristics of cytidine (and partly uridine and adenosine) transport, its kinetic parameters and specificity.

MATERIALS AND METHODS

Organisms: Prototrophic strain *Bacillus subtilis* SMYW was the only bacterial strain used.

Chemicals: Cytidine and xanthosine were purchased from Lachema (Brno), cytosine from Light & Co, Ltd., uridine from Sigma Chemical Company, uracil from Chemapol, 2'-deoxyuridine from Cambrian Chemicals Ltd., adenine, adenosine, 2'-deoxythymidine, guanosine, 2'-deoxycytidine and 2'-deoxyadenosine (all A grade) were from Calbiochem. All the other analogues of pyrimidine and purine nucleosides were synthesized and kindly provided by colleagues from the Department of Organic Synthesis of our Institute. The labelled substrates ^3H -cytidine and ^{14}C -uridine were supplied by the Institute for Research, Production and Application of Radioisotopes (Czechoslovakia) and ^3H -adenosine by the Radiochemical Centre (Amersham). The purity of all chemicals was checked up by paper chromatography using n-butanol-water system.

Measurement of the uptake velocities of the labelled compounds: An overnight culture of *B. subtilis* SMYW grown in Spizizen's ^{13}C minimal medium with glucose at 37°C was centrifuged and resuspended in the same fresh medium. After dilution to approximate density of 10^8 cells/ml the cultivation continued till the late logarithmic phase of growth. The cells were then washed with minimal medium and resuspended in a fresh Spizizen's medium containing glucose (0.5%) and chloramphenicol ($100\text{ }\mu\text{g/ml}$). The density of the bacterial culture was $5 \cdot 10^8 - 10^9$ cells/ml. After 15-30 min of preincubation at 37°C 0.8 ml aliquots from this suspension were pipetted into test-tubes containing 0.1 ml of radioactive nucleoside at appropriate concentration and specific activity and 0.1 ml either of a solution of the examined nucleoside or of minimal medium. The uptake was terminated after 60 sec by filtering the suspension through Sartorius membrane filter (pore size $0.2\text{ }\mu$) and washing twice with 10 ml of room-warm Spizizen's medium without glucose.

The time necessary to process all samples did not usually exceed 40 min. In the course of experiments several uninhibited parallel samples were always included to check the invariability of uptake. In each experiment the blank values of radioactivity were obtained by filtering and washing the same amounts of radioactive solutions without bacteria. These values were subtracted from all experimental values. Radioactivity on the membrane filters was measured in the Packard Tricarb (model 3375) scintillation counter using a toluene scintillation liquid.

Assays of labelled compounds in soluble pool of bacteria:

Membrane filters with the collected cells with retained radioactivity were suspended in 5.0 ml of water and heated in a boiling water bath for 5 min. The suspension was cooled down, filtered through membrane filters and washed twice with 2.0 ml of water. The filtrate alone with washings were dried, redissolved and separated chromatographically.

RESULTS

The active nature of cytidine transport in *B. subtilis* has been proved by following findings:

- 1) Initial rates of uptake obeyed saturation kinetics. The apparent affinity constant (K_m) for the uptake of cytidine was estimated to be $7.1 \mu M$. This average value was taken from four experiments.
- 2) The transport of cytidine in *B. subtilis* is sensitive to energy poisons. In bacterial suspension preincubated for 30 min in glucose minimal medium with either sodium azide ($5 \times 10^{-2} M$) or 2,4-dinitrophenole ($10^{-2} M$), no uptake of radioactive cytidine was observed.
- 3) The transport of cytidine was markedly reduced (to about 15%) when no external sugar was present. The uptake was stimulated by D-forms of ribose, xylose, glucose, mannose, galactose, fructose and by disaccharides saccharose, trehalose and maltose. Arabinose, lyxose, allose and altrose were without effect.

The fate of cytidine after uptake into cells: After 60 sec of incubation with 3H -cytidine 85-90% of the total radioactivity was recovered as TCA soluble material. Low-molecular-weight compounds extracted by boiling the bacteria in water

were separated by descending chromatography on Whatman No. 1 paper. In the systems butanol-water (86:14, by vol.) and methanol-conc. HCl-water (70:20:10, by vol.) the major part of radioactivity remained at the origin. In the system isobutyric acid-conc. ammonium hydroxide-water (66:1.5:33) radioactivity moved as a broad peak corresponding to mono-, di- and triphosphates of cytidine and uridine. Detailed quantitative and qualitative identification was not performed.

Furthermore, the efflux of radioactivity from bacteria was followed. The cells with accumulated radioactivity originating from ^3H -cytidine (20 μM , 100 $\mu\text{Ci}/\mu\text{mole}$, 120 sec of uptake) were washed on membrane filters and resuspended in glucose minimal medium with or without addition of non-radioactive nucleoside (100 μM). Radioactivity in the cells dropped approximately by 20% in the presence of external cytidine or uridine during 20 min but no decrease of retained radioactivity was observed in pure glucose minimal medium or in media with adenosine and guanosine, respectively.

Specificity of the cytidine-transport system: Free pyrimidine and purine bases and their 5'-nucleotides did not interfere with the cytidine-uptake; however, this uptake was significantly influenced by a number of pyrimidine ribonucleosides and 2'-deoxyribonucleosides and also by adenosine and 2'-deoxyadenosine. On the other hand, guanosine and xanthosine were without inhibitory effect. When studying the action of inhibitory nucleosides, both Lineweaver - Burk's¹⁴ and Dixon's¹⁵ plots were used. While all double reciprocal plots were linear and corresponding to the competitive case of inhibition, the Dixon's plots for 2'-deoxyribonucleosides were hyperbolic (Fig. 1). This result corresponds to the so-called apparently competitive or hyperbolic competitive type of inhibition^{16,17}. When uridine (50-200 μM) was added to ^3H -cytidine with 500 μM 2'-deoxycytidine, the slopes of the Dixon's inhibition curves changed abruptly instead of tending to level off.

In addition to cytidine, the transport of ^{14}C -uridine and ^3H -adenosine and their mutual influence on the uptake were studied. The results are presented in Tab. 1. Cytidine in-

Fig. 1 Nucleosides inhibiting ^3H -cytidine transport into *Bacillus subtilis*

Inhibitor	Shape of inhibition curves
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s_1, s_2 : conc. of ^3H -cytidine ($2\mu\text{M}$, $8\mu\text{M}$; $100\mu\text{Ci}/\mu\text{mole}$)

i : conc. of inhibiting cold nucleoside ($0-200\mu\text{M}$)

v : initial rate of uptake

hibited competitively the transport of uridine and vice versa. K_m 's for uptake of cytidine and uridine were not significantly different from the inhibition constants of the same substance used as an inhibitor of the other. Adenosine inhibited both cytidine- and uridine-uptakes with practically the same K_i , whereas these nucleosides did not influence adenosine-transport. K_m for adenosine differed markedly. Therefore, it can be assumed that cytidine and uridine are transported by a common transport process. Adenosine has an affinity for this transport site but requires a different site for its own transport.

Table 1 Apparent affinity (K_m) and inhibition (K_i) constants of cytidine, uridine and adenosine for their uptake systems in *B. subtilis*

Labelled substrates	$K_m (\mu\text{M})$	$K_i (\mu\text{M})$ on uptake of		
		cytidine	uridine	adenosine
Cytidine	7.1 ± 0.24	-	6.3	6.8
Uridine	8.1 ± 0.36	6.7	-	7.0
Adenosine	1.8 ± 0.08	no effect	no effect	-

When investigating effects of other pyrimidine nucleosides on the uptake of ^3H -cytidine we could assume further characteristics and specificity of a common cytidine- and uridine-uptake system (see Tab. 2). None of the arabinosides tested was an inhibitor of cytidine-uptake and therefore, the presence of either ribose or 2'-deoxyribose moiety in nucleosides might be necessary for their affinity to this transport system. 2,2'-Cycloforms and 6-azaderivatives (in contrast to 5-azaderivatives) did not interfere with the cytidine-uptake. Of course, gross rearrangements of pyrimidine base in nucleosides are not compatible with the affinity to transport system in question.

Table 2 Pyrimidine nucleosides not markedly interfering with cytidine-uptake

Cytosine arabinoside	Cyclocytidine	6-Azacytidine	Orotidine
Isocytosine arabinoside	Cyclouridine	6-Azauridine	Pseudouridine
Uracil arabinoside			Showdomycin
			Maleic acid hydrazide-ribose

All compounds listed in Tab. 2 were tested up to the 1000 μM concentration, which was 125 times and 500 times higher than that of labelled substrate.

DISCUSSION

In the above reported experiments the cellular retention of radioactivity from cytidine was used as a measure of cytidine transport. According to Von Dippe⁸ such results can be questioned for several reasons:

- 1) Nucleosides might undergo a cleavage and the uptake of radioactivity from base-labelled nucleosides into the cells might reflect transport of the base in addition to the intact nucleosides.
- 2) Transported nucleoside might be partially converted to the base and excreted and thus, the retention of radioactivity may give a false value for transport.
- 3) Intracellular conversion of nucleoside might influence the cellular uptake.

However, the cytidine-uptake in our experiments was not influenced by cytosine. We can therefore assume that in this case only nucleoside-uptake was followed and exclude the first objection. Further, no excretion of radioactivity in glucose minimal medium was found. The rate of uptake might be influenced by utilization of labelled substrate into polynucleotides. Only about 10% of labelled taken up substrate was found as a TCA precipitable material. However, the maximum velocity changed in different cultures, while the K_m values and patterns of inhibitory effects were reproducible.

The radioactivity originating from cytidine in the soluble pool belonged to phosphorylated products mainly. However, we cannot conclude that the whole flow of cytidine and uridine is directly coupled to phosphorylation. In such a case we could not observe any countertransport. But it is questionable whether the observed decrease of the accumulated radioactivity as low as 20% during 20 min represents a true countertransport phenomenon.

Only nonlabelled cytidine and uridine caused a decrease of radioactivity retained by cells; adenosine, which also competed with the uptake of cytidine, did not exhibit this effect. This supports the idea that adenosine competes only for a recognition site of cytidine- and uridine-uptake system, but in itself it is transported by additional different system(s). Our preliminary results showed further differences of adenosine uptake: it is influenced by adenine and, to a lesser extent, by guanosine; the Dixon's plot is linear when 2'-deoxyadenosine is used as an inhibitor. A similar anomaly in the transport relationship between adenosine and pyrimidine nucleosides exists also in *E. coli* B: adenosine inhibits transport of uridine and cytidine, whereas these nucleosides do not inhibit adenosine-transport⁷. However, the uridine-uptake was not influenced by adenosine; this observation is at variance with our results in *B. subtilis*. Komatsu^{4,5} proved that 2'-deoxycytidine transport in *E. coli* K-12 is mediated by two different systems: one mostly inhibited by adenine- and pyrimidine nucleosides, and slightly inhibited by guanine- and hypoxanthine nucleosides and

the other one which is strongly inhibited both by pyrimidine and all purine nucleosides. Doskočil¹⁰ brought an evidence of cytidine transporting systems in *E. coli* with low and high affinities to cytidine.

The existence of more than one transporting system with different affinities and specificities for a nucleoside, e.g. a common system for a wide variety of nucleosides and a more specific system for a respective nucleoside makes possible the development of resistance to structural analogues by an alteration or a loss of one of these systems. None of our results brought any convincing evidence of the presence of more than a single transport-system for cytidine and uridine in *B. subtilis* as it was reported for *E. coli*. The linearity of the Lineweaver - Burk's plot does not contradict such possibility¹⁸. Our so far tested mutants¹⁹ R_0 and R_2 , resistant to 5-azacytidine (an antimetabolite which competitively inhibits the cytidine-uptake, see Fig. 1) and one mutant resistant to 2'-deoxy-5-azacytidine showed exactly the same affinity constant for cytidine-uptake as the parent strain. In R_0 and R_2 mutant strains 5-azacytidine inhibited the cytidine-uptake with the same affinity as in the parent SMYW strain.

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