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THE COMPONENTS OF THE NUCLEOSIDE-TRANSPORTING SYSTEM IN *ESCHERICHIA COLI*

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SUMMARY

A mutant of *Escherichia coli* resistant to 5-azacytidine was selected and shown to be deficient in the high-affinity component of the nucleoside-transporting system which participates in several types of conversion of nucleosides, such as incorporation and deamination of 5-azacytidine and cytidine as well as phosphorolysis of thymidine. All these reactions are affected in a similar manner when wild-type cells are infected with T4-phage or treated with osmotically disrupted phage, while the treatment of mutant bacteria with this phage does not significantly alter the value of the Michaelis constant for the phosphorolysis of thymidine. Since the metabolic conversion of nucleosides in mutant cells remains susceptible to competitive inhibition with heterologous nucleosides, it seems that another, low-affinity component participates in the transport, which is still active in mutant or phage-infected bacteria. Cytidine is transported predominantly by the low-affinity component, provided its concentration in the medium is sufficiently high; 5-azacytidine, however, requires the high-affinity component for efficient transport at any concentration.

INTRODUCTION

The transport of many nutrients and metabolites across the cell membrane is mediated by specific systems which may consist of several components with different functions and range of specificity[1-3]. Although the transport of pyrimidine bases and nucleosides has been studied extensively in yeast cells[4], little is known about nucleoside permeases in bacteria[5,6]. Selection for resistance to structural analogues of metabolites has often been used as a means of obtaining mutants deficient in specific permeases[7-10]. 5-Azacytidine, a structural analogue of cytidine, competitively inhibits the metabolic conversion of other nucleosides[11], presumably competing for a component of the nucleoside–permease system. We expected that deficiency in this component might render the cells resistant to 5-azacytidine. Therefore, we isolated a set of mutants resistant to 5-azacytidine and compared the kinetics of metabolic conversion of several nucleosides by mutant and wild-type cells.

MATERIALS AND METHODS

The following non-labeled nucleosides were used: Cytidine, Hoffmann La Roche; adenosine, E. Merck; deoxyadenosine, chemically pure, Fluka; thymidine, A-grade, Calbiochem. 5-Azacytidine was prepared in this institute using the methods described previously[12]. [2-^{14}C]Thymidine (specific activity, 40.5 mCi/mmole), [G-^{14}C]-cytidine (210 mCi/mmole) and [8-^{3}H]adenosine (8 Ci/mmole) were obtained from the Institute for Research and Application of Radioisotopes, Praha. [4-^{14}C]5-Azacytidine (30 mCi/ mmole) was prepared in this institute by Ing. J. Kopecký.

Spontaneous mutants resistant to 5-azacytidine were selected as follows: A culture of the parent strain, *E. coli* B, diluted to $5 \cdot 10^5$ cells/ml, was plated on minimal glucose–mineral salts agar[13] containing 10 μg/ml 5-azacytidine. In order to avoid decomposition of 5-azacytidine by heat, this substance was added to the agar medium cooled down to about 50 °C just before pouring the plates. Wild-type bacteria did not form visible colonies on these plates even after 3 days incubation at 37 °C, while strains resistant to 5-azacytidine grew with some delay, forming colonies after about 36 h. The mutants retained their resistant character during many passages on tryptone agar without 5-azacytidine.

The bacteria were grown in shaken flasks at 37 °C on glucose–mineral salts medium[13] supplemented with 0.25 % casamino acids. For measurements of velocities of metabolic conversion of nucleosides the logarithmically growing bacteria at a density of about $3 \cdot 10^8$ cells/ml were filtered on a nitrocellulose membrane filter (Sartorius, diameter 50 mm) and resuspended in fresh medium with glucose but without casamino acids. For most kinetic measurements the density of $5 \cdot 10^8$ cells/ml was suitable; if data at very low substrate concentrations were required, the bacteria were diluted to $5 \cdot 10^7$ cells/ml. All data concerning the maximum reaction velocities (Table I) have been normalized to $1 \cdot 10^8$ cells/ml, assuming a linear relationship between the reaction rate and the number of bacteria per unit volume.

The preparation of T4-phage stocks and the inactivation of phage by osmotic shock has been described elsewhere[14]. For infection 10 plaque-forming units of phage, or 20 disrupted phage particles per bacterial cell were used. 3 min after the addition of phage, samples were taken for determining the number of surviving cells, and the reaction was started by adding the labeled nucleosides.

The rate of incorporation of nucleosides was measured by taking samples (1–2 ml) into an equal volume of 10 % trichloroacetic acid, filtrating the mixture on nitrocellulose membrane filters, and counting on a gas-flow counter. Other types of metabolic conversion of nucleosides were investigated in the following manner: At zero time aliquots of suitably diluted bacterial culture were pipetted into prewarmed shaken small erlenmeyer flasks containing the labeled substrates and inhibitors dissolved in a minimum volume of water. Samples (0.25 ml) were usually taken at 4-min intervals into chilled tubes containing excess of non-labeled substrate and products of the reaction under study. The samples were analyzed by paper chromatography as described previously[11]. In the simplest cases, such as the incorporation of cytidine or 5-azacytidine, the reaction velocity was constant for at least 20 min. The phosphorolysis of thymidine was usually more rapid during the first 3–4 min than afterwards; in such cases the rate between 4 and 20 min after the addition of the substrate was taken as the reaction velocity. The deamination of adenosine[15] in

the mutant strain took a very complex course due to the rapid exhaustion of the substrate by incorporation into RNA, as well as by further conversion of primarily formed inosine; therefore, meaningful quantitative data about the kinetics of this reaction could not be obtained.

The rate of deamination of 5-azacytidine could not be determined by chromatography since the 5-azauridine formed is very unstable in neutral aqueous solution[16]. 5-Azacytidine and 5-azauridine in acidic media have absorption maxima at 255 and 240 nm, respectively; therefore, qualitative evidence of deamination could be obtained in glucose–mineral salts medium (without amino acids), filtered on nitrocellulose membrane filters to remove bacteria and acidified to pH 1.0 with 2M HCl, measuring the shift of the absorption maxima from 255 nm to shorter wavelengths.

RESULTS

Spontaneous mutants resistant to 5-azacytidine were found to occur in populations of *E. coli* at a frequency of about 10^{-4}. Each of the eighteen resistant strains isolated had approximately the same degree of tolerance of the inhibitor, their growth being partially retarded by 10 μg/ml of the inhibitor in the agar medium. All mutants have been shown to incorporate 5-azacytidine at a reduced rate in comparison with the wild-type strain. One of these mutants was investigated more closely, determining the kinetics of metabolic conversion of various nucleosides by whole cells.

Lineweaver–Burk plots of the concentration dependence of the rates of incorporation of both cytidine and 5-azacytidine showed that the Michaelis constant for the incorporation of both substrates is markedly increased in the mutant when compared with wild-type strain. In addition, the maximum velocity (V) of 5-azacytidine incorporation is about 6 times lower in the mutant than in the wild-type strain (Fig. 1B) while there is no significant difference in the V value of cytidine incorporation (Fig. 1A). Consequently, the resistant mutant incorporates much less 5-aza-

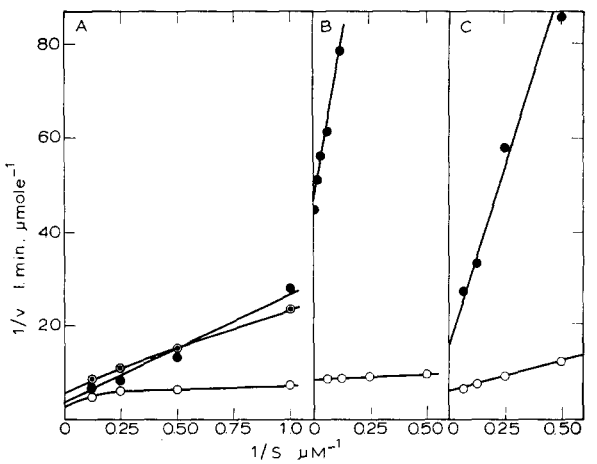

Fig. 1. Kinetics of metabolic conversion of cytidine and 5-azacytidine by strains of *E. coli*, sensitive and resistant to 5-azacytidine. A, incorporation of cytidine; B, incorporation of 5-azacytidine; C, deamination of cytidine. ○—○, *E. coli* B (wild-type strain); ◉—◉, *E. coli* B, infected with T_4-phage; ●—●, 5-azacytidine-resistant strain. The density of the bacterial suspension was $5 \cdot 10^7$ cells/ml.

cytidine than the wild-type strain, irrespective of substrate concentration in the medium; with cytidine a marked difference in the incorporation rates is detectable at concentrations of about 1 μM; when the substrate concentration is increased up to 8 μM the difference becomes less apparent.

The deamination of cytidine is affected in the mutant strain in a similar manner; Fig. 1C shows a considerable increase of the Michaelis constant and some reduction of maximum velocity in the mutant. The kinetics of deamination of 5-azacytidine could not be measured exactly, since the 5-azauridine formed is rapidly decomposed. Spectrophotometric data show, however, that 5-azacytidine is stable in contact with mutant cells, while a gradual shift of absorption maxima at pH 1.0 is observed in the wild-type culture, indicating deamination of 5-azacytidine.

The pleiotropic character of the mutation leading to resistance to 5-azacytidine is illustrated by the fact that phosphorolysis of thymidine, a reaction apparently unrelated to the metabolism of cytidine, was affected in a similar manner; we observed a marked increase of Michaelis constant with little change of maximum velocity (Fig. 2).

Many attempts were undertaken to measure the rates of deamination of adenosine by mutant and wild-type cells; no exactly comparable data could, however, be obtained for reasons explained above; nevertheless the experiments (not shown) indicated that the difference between both strains is much smaller than in the case of deamination of cytidine or phosphorolysis of thymidine.

A peculiar property of mutant cells, *i.e.* the strong impairment of the ability to incorporate 5-azacytidine concurrent with a moderate reduction of cytidine uptake, strikingly resembles the effect of T4-phage infection on wild-type cells[14]. In order to verify the significance of this similarity we repeated some of the foregoing experiments now comparing the intact wild-type bacteria with those infected with T4-phage or treated with disrupted phage. We observed that the kinetics of these reactions was altered by phage; the Lineweaver–Burk plots of infected wild-type cells were nearly superimposable with those of the mutant, but divergent from those of the uninfected culture (Figs 1 and 2). It seems that the mutation had affected the same catalytic

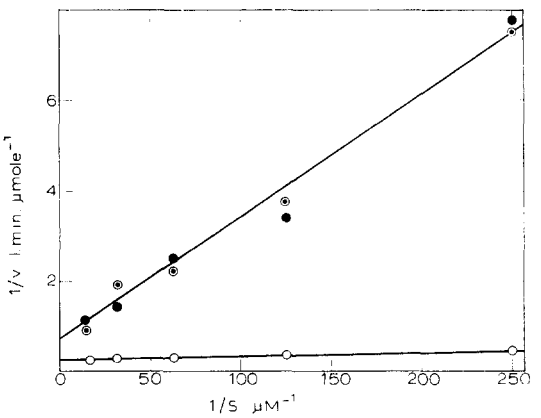

Fig. 2. Kinetics of phosphorolysis of thymidine by strains of *E. coli*, sensitive and resistant to 5-azacytidine. ○—○, *E. coli* B (wild-type strain); ◉—◉, *E. coli* B, treated with osmotically disrupted T4-phage; ●—●, 5-azacytidine-resistant strain. The density of the bacterial culture was $5 \cdot 10^8$ cells/ml.

component of the cell as does the phage; if this is correct, we would expect that the kinetic characteristics of the mutant would not change any further upon phage treatment. The data of Table I show that the K_m of phosphorolysis of thymidine increases 8.5 times upon treatment of wild-type cells with disrupted phage, while a similar treatment of mutant strain causes a relatively small increase by a factor of 1.5. The observed decrease of V may perhaps be due to blocking of the catabolism of the deoxyribose moiety of thymidine as observed by Munch-Petersen and Vilstrup[17]. It should be noted that the mutant is equally sensitive to T4-phage as the wild-type strain, propagating this phage with equal efficiency.

TABLE I

APPARENT MICHAELIS CONSTANTS AND MAXIMUM REACTION VELOCITIES OF METABOLIC CONVERSION OF CYTIDINE AND 5-AZACYTIDINE BY 5-AZACYTIDINE-SENSITIVE (WILD-TYPE) AND RESISTANT STRAINS OF *E. coli*

The values of maximum reaction velocities have been normalized to a density of bacterial culture equal to $1 \cdot 10^8$ cells/ml.

Substrate	*Reaction*	*Bacteria*	V ($\mu moles \cdot min^{-1} \cdot l^{-1}$)	K_m (μM)
Cytidine	Incorporation	Wild-type	0.36*	0.25
	Incorporation	Resistant	0.56	7.1
	Incorporation	Wild-type, T4-infected	0.42	4.2
5-Azacytidine	Incorporation	Wild-type	0.28	2.1
	Incorporation	Resistant	0.05	20
Cytidine	Deamination	Wild-type	0.34	2.3
	Deamination	Resistant	0.14	8.7
Thymidine	Phosphorolysis	Wild-type	0.83	4.0
	Phosphorolysis	Resistant	0.27	34
	Phosphorolysis	Wild-type, T4-infected	0.27	34
	Phosphorolysis	Resistant, T4-infected	0.15	50

* Value obtained by extrapolating the less steep straight line (Fig. 1A) to zero reciprocal substrate concentration.

The foregoing experiments indicate that the mutation and the treatment with T4-phage affect a high-affinity component common to incorporation or deamination of cytidine and 5-azacytidine as well as to the phosphorolysis of thymidine.

In wild-type cells many types of metabolic conversion of nucleosides are competitively inhibited by structurally and metabolically unrelated nucleosides[5,6,11] and it was of interest to investigate whether the low-affinity conversions in mutant bacteria are still susceptible to this kind of inhibition. Therefore, the competition experiments were repeated with the mutant strain, and the results were expressed in terms of the K_m/K_i ratios (Table II), calculated from the Lineweaver–Burk plots as earlier described[11]. Fig. 3 shows that 5-azacytidine as well as cytidine are capable of competitively inhibiting the phosphorolysis of thymidine in the mutant; the K_m/K_i ratios are close to unity; in fact, 5-azacytidine is slightly more active than cytidine, in spite of the very limited rate of uptake of the former. This finding again confirms the parallelism between the mutated and T4-phage-infected cells, where 5-azacytidine

TABLE II

THE VALUES OF K_m/K_i OF NUCLEOSIDE CONVERSION INHIBITED BY HETEROLOGOUS NUCLEOSIDES

Substrate	*Reaction*	*Inhibitor*	K_m/K_i	
			Wild-type bacteria*	*Mutant bacteria*
Thymidine	Phosphorolysis	Cytidine	n.d.**	1.03
	Phosphorolysis	5-Azacytidine	3.5	1.23
5-Azacytidine	Incorporation	Adenosine	1.32	1.34
	Incorporation	Deoxyadenosine	n.d.**	0.45
	Incorporation	Thymidine	0.25	0.24

* The values for wild-type bacteria are taken from ref. 11.
** n.d., not determined.

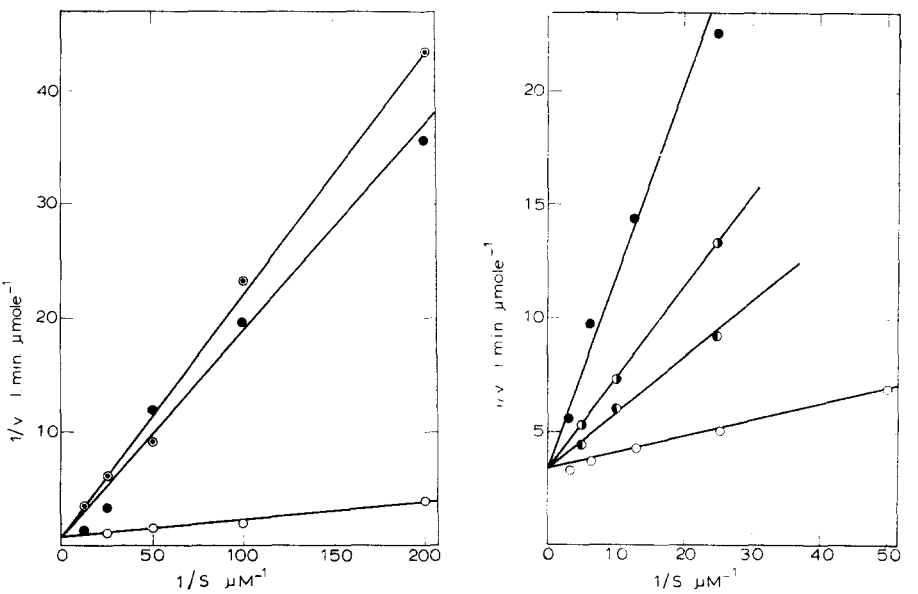

Fig. 3. Inhibition of phosphorolysis of thymidine in 5-azacytidine-resistant mutant by cytidine and 5-azacytidine. ○—○, thymidine alone; ●—●, cytidine; ◎—◎, 5-azacytidine. The concentration of competing nucleosides was 200 μM. The density of the bacterial cultures was $5 \cdot 10^8$ cells/ml.

Fig. 4. Inhibition of incorporation of 5-azacytidine in 5-azacytidine-resistant mutant by heterologous nucleosides. ○—○, 5-azacytidine alone; ●—●, adenosine; ◑—◑, deoxyadenosine; ◐—◐, thymidine. The concentration of the competing nucleosides was 200 μM. The density of bacterial culture was $7.5 \cdot 10^8$ cells/ml.

retains its ability to inhibit the phosphorolysis of thymidine in spite of selective impairment of its uptake[18,19]. The residual incorporation of 5-azacytidine by the mutant strain is still competitively inhibited by adenosine and less efficiently by deoxyadenosine and thymidine (Fig. 4).

DISCUSSION

Selecting for resistance to 5-azacytidine, a strain of *E. coli* was obtained with altered kinetics of metabolic conversion of nucleosides, involving at least three distinct enzymes, namely cytidine kinase (EC 2.7.1.48), cytidine deaminase (EC 3.5.4.5) and thymidine phosphorylase (EC 2.4.2.4). Except for a common nucleoside or deoxynucleoside structure of substrates, these reactions are apparently unrelated; the impairment of the rate of incorporation of 5-azacytidine cannot be due to a lack of deamination, since we know that strains deficient in cytidine deaminase incorporate 5-azacytidine with nearly the same efficiency as wild-type strains[20]. All enzymes are still functional in the mutant and probably also in T4-treated cells, since the reactions catalyzed by them proceed at sufficiently high concentrations of the substrates. Munch-Petersen and Vilstrup[17] measured the activities of enzymes of thymidine catabolism in cell-free extracts of bacteria before and after infection with T4-phage or treatment with phage ghosts; no decrease of the activity of thymidine phosphorylase was found. It seems that the mutation as well as the phage treatment affect a catalytic component common to all these enzymic conversions, which is most likely to be a permease mediating the transport of several nucleosides and deoxynucleosides. The existence of such a permease has been anticipated on the basis of mutual competition of heterologous nucleosides[5,6]. The present experiments show, however, that even if the high-affinity component is missing, the residual low-affinity reactions are still susceptible to competitive inhibition by heterologous nucleosides. Therefore, wild-type bacteria seem to contain two different nucleoside permeases with different affinity for the substrates. The high-affinity component is inactivated by the mutation or T4-phage treatment, while the low-affinity component remains operative. Both these permeases are common for a number of different nucleosides and deoxynucleosides; no permease specific for a single nucleoside has been found so far.

It may be objected that neither the competition of heterologous nucleosides nor the pleiotropic character of a mutant necessarily indicate the involvement of permeases. Hammer-Jespersen *et al.*[21] found that adenosine or cytidine induce the synthesis of thymidine-catabolizing enzymes; no explanation has been proposed. This remarkable finding shows that apparently independent pathways of nucleoside metabolism may actually be linked by unknown catalytic or regulatory components. In case of our mutant, however, in which the kinetics of different types of metabolic conversion is affected in an analogous manner, the permease hypothesis seems to be the most acceptable.

The existence of two distinct permeases enables us to explain the differential effect of the mutation or phage treatment on the incorporation of cytidine and 5-azacytidine. We have seen that the high-affinity component of cytidine incorporation is very limited, being detectable at very low substrate concentrations only; at higher concentrations most of the cytidine incorporated is transported by the low-affinity component. The opposite is true with 5-azacytidine; its transport by the low-affinity system is very inefficient and practically all uptake in the wild-type strain is due to the high-affinity permease. Therefore, the inactivation of the latter strongly affects the incorporation of 5-azacytidine but has little effect on the incorporation of cytidine, unless measured at very low substrate concentration. The heterogeneity of cytidine transport in *E. coli* has been postulated earlier by Peterson *et al.*[5]. It is remarkable

that 5-azacytidine very efficiently inhibits the transport mediated by the low-affinity permease but that its own transport by this permease is severely limited. No definite explanation for this behaviour can be proposed at present. Many examples of this type of interaction are known from studies of the permeases of amino acids[22].

We may ask whether the two permease components are acting independently in parallel or whether they operate in a consecutive manner, forming an integrated system as proposed by Koch[3].

According to Peterson and Koch[6] the nucleoside-transporting system consists of a binding site, a permease and possibly a component mediating the transfer of energy. The histidine-transporting system in *Salmonella typhimurium* has been shown[23] to consist of two distinct independent binding proteins of high affinity, and a permease; an additional, less specific transport system may participate in histidine uptake, working in parallel with the main histidine-specific system. The relationship between the two nucleoside-transporting components cannot be determined with certainty until mutants deficient in the low-affinity component become available; obviously such mutants cannot be expected to occur among 5-azacytidine-resistant strains; slowly growing cytidine auxotrophs should be promising for the isolation of this kind of mutants.

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