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TRANSPORT OF URIDINE IN *ESCHERICHIA COLI* B AND A SHOWDOMYCIN-RESISTANT MUTANT

P. J. VON DIPPE, S. ROY-BURMAN and D. W. VISSER

Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, Calif. 90033 (U.S.A.)

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SUMMARY

The mechanism of uridine transport in *Escherichia coli* B cells was studied using experimental approaches designed to limit possible ambiguities in interpretation of data obtained previously. For this purpose, the transport of [2-^{14}C]uridine and [U-^{14}C]uridine was determined in *E. coli* B and an *E. coli* B mutant which is resistant to the inhibitory effects of the nucleoside antibiotic, showdomycin.

The majority of the uridine transported as the intact nucleoside is cleaved to uracil and ribose 1-phosphate. The uracil, in large part, is excreted, while ribose 1-phosphate is retained. In addition, uridine is also rapidly cleaved to uracil and ribose 1-phosphate in the periplasmic space. The uracil moiety may enter the cell, whereas ribose 1-phosphate is not transported. The showdomycin-resistant mutant transports the intact nucleoside inefficiently, or not at all, but retains its ability to convert uridine to uracil in the periplasmic space.

INTRODUCTION

Current concepts of nucleoside transport mechanisms in *Escherichia coli* have been based on data gathered from two general experimental approaches. Several studies[1–3], including previous work in this laboratory, have utilized the cellular retention of radioactivity from nucleosides as a measure of nucleoside transport. Although this approach provides data which are suggestive of certain conclusions regarding nucleoside transport, the validity of the conclusions may be questioned for several reasons. For example, Peterson *et al.*[4] have reported that uracil rapidly appears in the medium in relatively large amounts as compared to cellular retention when *E. coli* are incubated with uridine or cytidine. Therefore, the uptake of radioactivity from base-labeled nucleosides into cells may reflect transport of uracil in addition to the intact nucleosides. In addition, since transported uridine may be partially converted to uracil and excreted, retention of radioactivity may give a false value for transport. In our studies, nucleosides labeled in the base moiety were employed, except for cytidine, which was uniformly labeled. Retention of uniformly labeled cytidine is about 6-fold greater than retention of other nucleosides, indicating a possibility that the ribose portion of the molecule is retained to a greater extent than the base moiety. Furthermore, it is difficult to quantitate the extent to which the rate

of intracellular conversion of the free base or nucleoside into the nucleotide pools and nucleic acids influences the observed cellular uptake at the variety of conditions used to measure nucleoside transport. A second approach[4,5] quantitates the appearance of uracil in the medium as a measure of uridine, cytidine or deoxycytidine transport into *E. coli* cells. The validity of this approach for measurement of nucleoside transport is dependent upon the assumption that all the uracil is formed from the nucleosides intracellularly and is subsequently excreted into the medium. This may not be a valid assumption, since several investigators[6,7] have reported the release of nucleoside phosphorylases from *E. coli* by the osmotic shock procedure of Nossal and Heppel[8]. Enzymes released in this fashion are assumed to be located in the periplasmic space or at the surface of the inner membrane. For example, Hochstadt-Ozer[6] has implicated the conversion of adenosine to adenine by adenosine phosphorylase (adenosine:orthophosphate ribosyltransferase EC 2.4.2.2) in isolated membrane vesicles as a prerequisite for the transport of adenosine. Therefore, it is important to establish the extent of these degradative processes which may occur in the periplasmic space before an accurate estimation of intact nucleoside transport can be made.

The present studies were undertaken to clarify the mechanisms utilized by *E. coli* B for the transport of uridine into the cell, utilizing experimental approaches designed to circumvent some of the above difficulties in interpretation of data. For this purpose the transport of both [2-^{14}C]uridine and [U-^{14}C]uridine was determined in *E. coli* B and an *E. coli* B mutant which is resistant to the inhibitory effects of the nucleoside antibiotic, showdomycin. The two radioactive substrates were used as a means for identification of possible contributions of extracellular and intracellular reactions. The showdomycin-resistant mutant was employed, since previous work showed that the mutant has a decreased capacity to transport showdomycin as well as naturally occurring nucleosides[3]. Therefore, differences in the observed uptake by the mutant and wild-type cells were thought to offer an additional means for distinguishing nucleoside transport from other processes such as the transport of uracil which is formed from uridine in the periplasmic space.

MATERIALS AND METHODS

Materials

The ^{14}C-labeled compounds were obtained from New England Nuclear.

Isolation of showdomycin-resistant mutants

An *E. coli* B (Hill) mutant, resistant to 80 μM showdomycin, was developed as described previously[3]. The mutant and the parent cells used in all experiments were grown in minimal medium[9] in the absence of showdomycin, and were harvested as described previously[3].

Assay for uptake

Unless otherwise noted, the standard reaction mixture (1 ml) contained 0.25 mM [^{14}C]uridine or [^{14}C]uracil and a cell suspension of *E. coli* B, or the showdomycin-resistant mutant (equivalent to 0.4–0.5 mg dry weight) in Medium A*. The specific activities of uridine and uracil used were as follows: [2-^{14}C]uridine (0.8 Ci/mole);

* Minimal medium described by Davis and Mingioli[9] without glucose.

[U-^{14}C]uridine (0.4 Ci/mole or 0.9 Ci/mole); and [2-^{14}C]uracil (0.8 Ci/mole). After incubation at 37 °C in the presence of radioactive uridine or uracil for the indicated time period, the uptake was measured by rapid millipore filtration technique as described previously[3]. The measurements of radioactivity were carried out in a Beckman liquid scintillation spectrometer, Model No. LS 245, using a scintillation fluid containing 8 g of 2,5-diphenyloxazole and 20 ml of BBS-3 (Beckman Instruments) in 1 l of toluene. The data represent total retention of radioactive compounds in the cells after the indicated incubation period, including incorporation into acid-soluble compounds and nucleic acids.

Measurement of uracil, uridine and ribose 1-phosphate in the medium

Uracil and ribose 1-phosphate were measured in the filtrate after cells had been incubated under standard conditions for the indicated time period with [^{14}C]uridine and filtered on Millipore filters. The filtrate was lyophilized to dryness, and the residue dissolved in 1 ml of water; 0.1 ml was spotted on Whatman 3 MM paper, together with appropriate standard solutions; uracil, uridine and ribose 1-phosphate were separated by descending chromatography in butanol–water (86:14, by vol.). Fluorescent spots were visualized under ultraviolet light to locate the position of uracil and uridine. Ribose 1-phosphate remains at the origin in this system. Segments corresponding to uracil, uridine and ribose 1-phosphate were cut out and counted separately, as above, by liquid scintillation spectrometry. The identity of ribose 1-phosphate was confirmed as follows: The origin of a chromatogram of the filtrate developed as above was eluted with three 5-ml aliquots of water. The extracts were pooled and lyophilized. The residue was dissolved in a small volume of water, non-radioactive carrier ribose 1-phosphate was added, and the solution spotted on DEAE-cellulose paper (Whatman DE-81). The chromatogram was developed by descending chromatography in ammonium formate buffer (1 M ammonium formate in 4 M formic acid) for 4.5 h. A single radioactive peak was located on a radioactivity scanner. The radioactive area was extracted twice with 5 ml of water and the extracts pooled, lyophilized and dissolved in a small volume of 0.5 M HCL. After 30 min the solution was neutralized with 0.5 M NH_4OH, lyophilized, redissolved in water, and spotted on Whatman 1 MM paper alongside a standard ribose solution as a reference. The chromatogram was developed in isopropanol–butanol–water (10:10:20, by vol.) for 8 h, according to the method of Wood[11]. Ribose was located by spraying with triphenyl tetrazolium spray (Sigma Chemical Co.). A single radioactive peak was found at the same R_F value as standard ribose. The carrier ribose 1-phosphate produced a colored spot coinciding with the radioactivity at the R_F of ribose. Under these conditions, ribose 5-phosphate is not appreciably hydrolyzed and remains at the origin of the isopropanol–butanol–water chromatogram.

Analysis of the accumulated products of uracil and uridine uptake in E. coli B and mutant cells

The reaction mixtures (1 ml) containing a cell suspension of *E. coli* B, or the mutant (equivalent to 0.4 to 0.5 mg dry weight) in Medium A, were preincubated for 10 min at 37 °C prior to the addition of 0.25 mM [^{14}C]uridine or [^{14}C]uracil. About 4–7 μCi of ^{14}C-labeled substrate were used per experiment. After incubation for 5 min at 37 °C the reaction mixtures were chilled, diluted with 5 ml of ice-cold

Medium A, and filtered through Millipore filters (0.45 μm pore size), and the filters were washed three times with 5-ml portions of ice-cold Medium A. The Millipore filters with the collected cells were suspended in 4 ml of water and heated in a boiling water-bath for 2 min. The suspension was filtered through Millipore filters and washed with 2 ml of water. The filtrate together with the washings were lyophilized. The lyophilized products were separated by two chromatographic systems. In System I, the products were separated by descending chromatography on DEAE-cellulose paper with a solvent consisting of 0.1 M ammonium formate in 4 M formic acid. This system separates UMP, UDP and UTP, while uridine and uracil move together with the solvent front. In System II, the products were separated by descending chromatography on Whatman No. 3 MM paper with 86% *n*-butanol in water (v/v). In System II, R_F values for uridine and uracil were 0.17 and 0.31, respectively. The phosphorylated products remain at the origin.

Osmotic shock procedure and determination of uridine phosphorylase in shock fluid and cell homogenate supernates

The method for osmotic shock was that of Nossal and Heppel[8], with minor modifications. Cells were grown to early stationary phase in Davis and Mingioli medium[9]. Cells were harvested by centrifugation and washed three times with 10 mM Tris, 20 mM NaCl, pH 7.0. They were then suspended in 30 mM Tris, 1.0 mM EDTA, 20% sucrose, pH 8.0, to give a cell density of $5 \cdot 10^8$ cells per ml, and shaken gently by hand for 10 min at room temperature. The cells were centrifuged, the packed cells were resuspended by gentle shaking at 4 °C for 10 min in the same volume of ice-cold 0.5 mM $MgCl_2$, and centrifuged. The supernatant, termed the shock fluid, was filtered through Millipore filters to remove any cells.

Cell homogenates were prepared by suspension of early stationary phase cells in Davis and Mingioli[9] medium, to a density of $5 \cdot 10^8$ cells per ml. Cells were disrupted by sonic oscillation for 3 min.

Shock fluid and the supernatant from disrupted cells were assayed for uridine phosphorylase (uridine:orthophosphate ribosyltransferase EC 2.4.2.3) activity by incubation of 0.1 ml in the standard assay with [^{14}C]uridine. Uracil and ribose 1-phosphate were separated by paper chromatography in 86% butanol as above.

RESULTS

Table I shows retention of uracil and ribose moieties by *E. coli* B and mutant cells, and formation of uracil and ribose 1-phosphate in the medium, in the presence and absence of showdomycin. Retention of the uracil moiety was calculated from the retention of [2-^{14}C]uridine. The number of counts from [U-^{14}C]uridine corresponding to this value for the uracil moiety was calculated as indicated in the table. Total ribose incorporation was obtained by subtraction of the calculated uracil moiety counts from total counts incorporated from [U-^{14}C]uridine. The results indicate that the ribose moiety is retained to a greater extent than the uracil moiety by *E. coli* B and the mutant. Uracil retention by the mutant is about 50% of that retained by *E. coli* B. Ribose retention by the mutant is negligible. *E. coli* B cells produce approximately twice as much uracil as ribose 1-phosphate in the indicated time period. Addition of showdomycin decreases uracil production to the level of ribose 1-phosphate produc-

TABLE I

RETENTION OF URACIL AND RIBOSE MOIETIES OF URIDINE BY *E. COLI* B AND MUTANT CELLS, AND FORMATION OF URACIL AND RIBOSE 1-PHOSPHATE IN THE MEDIUM, IN THE PRESENCE AND ABSENCE OF SHOWDOMYCIN

Uptake of uracil and ribose 1-phosphate formation were measured after 10 min at 37 °C in the presence of either [2-^{14}C]uridine or [U-^{14}C]uridine under standard assay conditions except that prior to addition of ^{14}C-labeled nucleosides the reaction mixtures were preincubated for 10 min at 37 °C with or without showdomycin.

Strain	*Conditions*	*Inside the cell*		*In the medium*	
		*nmoles uracil**	*nmoles ribose***	*nmoles uracil*	*nmoles ribose 1-phosphate*
E. coli B	10-min incubation	2.0	32.4	108	48
	5-min incubation	1.1	17.6	54	24
	0.16 mM showdomycin (10 min)	0.85	10.0	50	49
	0.32 mM showdomycin (10 min)	n.d.***	n.d.	48	48
Mutant	10-min incubation	0.34	0.6	21.4	21.6
	0.16 mM showdomycin (10 min)	0.33	0.1	20.0	20.2
	0.32 mM showdomycin (10 min)	n.d.	n.d.	20.0	20.4

* a, Uracil moiety retained (obtained from retention of counts from [2-^{14}C]uridine).

** Ribose moiety retained was calculated as follows: b, cpm per nmole [U-^{14}C]uridine; c, cpm per nmole [U-^{14}C]uracil moiety = b × 4/9; d, cpm per nmole [U-^{14}C]ribose moiety = b × 5/9; e, cpm retained from [U-^{14}C]uridine. nmoles ribose moiety retained = e − (a × c)/d.

*** n.d., not determined.

TABLE II

EFFECT OF OSMOTIC SHOCK ON PRODUCTION OF URACIL BY *E. COLI* B AND MUTANT CELLS

Cells corresponding to 0.5 mg dry weight, or homogenate or shock fluid from this weight of cells, were incubated 5 min at 37 °C in the standard assay mixture with or without prior incubation with showdomycin.

Enzyme source	*% Uracil produced**			
	E. coli B		*Mutant*	
	No showdomycin	*0.32 mM showdomycin*	*No showdomycin*	*0.32 mM showdomycin*
Intact cells	100 (54)**	43 (23)	20 (11)	20 (11)
Shocked cells	80 (43)	24 (13)	8 (4)	8 (4)
Cell homogenate from intact cells	274 (148)	270 (145)	224 (112)	222 (112)
Cell homogenate from shocked cells	206 (111)	210 (112)	178 (96)	177 (95)
Shock fluid	53 (29)	53 (29)	50 (27)	50 (27)

* Values expressed as % of uracil produced by intact *E. coli* B cells (100%).

** Values in parentheses indicate nmoles of uracil produced.

tion, while having no effect on ribose 1-phosphate production. The mutant produces about one-fifth as much uracil as the wild type, and the concentrations of uracil and ribose 1-phosphate are equal. Showdomycin does not affect either uracil or ribose 1-phosphate production in the mutant.

The effect of osmotic shock and distribution of uridine phosphorylase activity is shown in Table II. The highest activity is obtained from disrupted cells. Osmotic shock decreases uracil production in the medium, and appreciable uridine phosphorylase activity is found in shock fluid. Although intact mutant cells produce less uracil than intact *E. coli* B cells, uridine phosphorylase activity of disrupted cells and in the shock fluid is very similar in mutant and *E coli* B.

Table III shows the distribution of radioactivity from radioactive uracil or uridine into nucleotides and uridine plus uracil. In both mutant and wild type, an appreciable amount of uracil plus uridine is present. System II, which separates uridine and uracil, shows an approximately equal distribution of nucleoside and free pyrimidine, and values for nucleotide retention agree closely with those obtained from System I.

TABLE III

DISTRIBUTION IN *E. COLI* B AND MUTANT CELLS OF RADIOACTIVITY FROM [2-^{14}C]URIDINE

System I separates UMP, UDP and UTP. Uracil and uridine move at the solvent front. Results obtained using System II, which separates uridine and uracil, are described in the text.

Strain	*% Total cpm, System I*	
	Nucleotides	*Uridine plus uracil*
E. coli B	60.6	40.6
Mutant	38.5	61.4

DISCUSSION

It is evident from Table I that the ribose moiety of uridine is retained to a far greater extent than the uracil moiety. This implies that uridine is cleaved after transport into the cell, presumably by uridine phosphorylase. A major portion of the uracil formed is excreted into the medium, whereas the ribose 1-phosphate is retained. The presence of ribose 1-phosphate in the medium at about 50% of the concentration of uracil (Tables I and II) indicates that *E. coli* B also cleaves uridine to uracil and ribose 1-phosphate in the periplasmic space. It may be concluded that about 50% of the uracil in the medium is formed intracellularly and excreted, and 50% is formed in the periplasmic space after a 10-min incubation period. This conclusion is supported by the demonstration of uridine phosphorylase activity in shock fluid (Table II), and is based on the reasonable assumption that ribose 1-phosphate is not readily transported in or out of the cell. It is possible that ribose 1-phosphate formed in the periplasmic space may be further metabolized to non-phosphorylated derivatives which are then transported into the cell, and thereby contribute to an apparent excess of the

ribose moiety over the uracil moiety of uridine in the cell. However, radioactive products other than ribose 1-phosphate are not found in the medium, and the addition of 2 mM ribose 1-phosphate has no effect on [2-^{14}C]uridine or [U-^{14}C]uridine retention by *E. coli* B, whereas 1.0 mM mannose produces about a 4-fold increase in [2-^{14}C]-uridine retention (unpublished data). If ribose 1-phosphate or a product of its metabolism were transported into the cell, it should be an efficient energy source and have an effect similar to mannose on retention of the uracil moiety of uridine. Moreover, as will be discussed below, data from the showdomycin-resistant mutant provide additional evidence against this as a likely mode of entry for ribose. A certain proportion of the ribose moiety is evidently lost from the cell, probably by degradation to CO_2. This effect is increased by longer incubation time since, after a 10-min incubation, the total nmoles of uracil moiety inside the cell *plus* uracil in the medium is greater by 30 nmoles than the total nmoles of ribose moiety in the cell *plus* ribose 1-phosphate in the medium. After a 5-min incubation the total uracil moiety inside and outside the cell is only 13 nmoles greater than the total ribose moiety inside and outside the cell (Table I). Since *E. coli* B cells retain the ribose moiety of uridine to a greater extent than the uracil moiety and cleave uridine to uracil in the periplasmic space, neither retention of counts from base-labeled uridine nor measurement of extracellular uracil can be taken as an accurate measure of uridine transport as the intact nucleoside. A better measure of transport appears to be retention of the ribose moiety of uridine, although even this measurement is complicated by a rapid decrease of extracellular uridine concentration.

The experimental approach used to obtain the data in Table I makes it possible to distinguish between the transport of intact uridine and the transport of uracil formed from uridine in the periplasmic space, a distinction not possible using former methods of transport analysis[2,3]. However, the present data are not in conflict with the conclusion drawn previously that the showdomycin-resistant mutant has lost the ability to transport uridine as the intact nucleoside[2,3]. In fact, the above conclusion is substantiated by the finding that very little of the ribose moiety of uridine appears in the mutant cell (Table I). This is evidence that the cellular radioactivity from the ribose moiety in *E. coli* B is indeed transported into the cell as uridine and is not the result of further metabolism of ribose 1-phosphate in the periplasmic space to compounds which are then transported into the cell. Degradation of uridine to ribose 1-phosphate in the periplasmic space occurs in both the mutant and *E. coli* B (Table I). Therefore, the possibility that ribose 1-phosphate is incorporated into *E. coli* B and not into the mutant would require that, in addition to loss of uridine transport, the mutant has lost either a transport system for metabolites of ribose 1-phosphate, or one or more enzymes of ribose 1-phosphate degradation.

The mutant has a diminished capacity to convert uridine to ribose 1-phosphate and uracil, but as discussed below, this may not result from a difference in uridine phosphorylase (Table I). The amount of ribose 1-phosphate appearing in the medium is not decreased in either *E. coli* B or the mutant by the presence of showdomycin. Thus, in the absence of uridine transport the production of uracil from uridine by *E. coli* B and the mutant is limited to the periplasmic space. It may also be concluded that showdomycin does not inhibit uridine phosphorylase in the periplasmic space. Additional evidence for this conclusion is apparent from the data of Table II, which show that showdomycin does not inhibit either intracellular or periplasmic uridine

phosphorylase. It is of interest in this regard that showdomycin does have a potent inhibitory effect on uridine phosphorylase from Ehrlich ascites cells[12].

The transport process for adenosine proposed by Hochstadt-Ozer[6] in *E. coli* K12, namely, an obligatory periplasmic phosphorolysis of adenosine to adenine, which is then converted to AMP by adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase EC 2.4.2.7) is not a likely mechanism for the transport of uridine in *E. coli* B. In addition to the evidence previously cited, indicating the transport of intact uridine as a major process, an appreciable amount of both uracil and uridine are present in cells (Table III). These data indicate the direct transport of intact uridine or uracil into the cell. In addition, Kornberg[10] and co-workers have reported that *E. coli* B does not contain uracil phosphoribosyltransferase (UMP: pyrophosphate phosphoribosyltransferase EC 2.4.2.9). For these reasons, a direct conversion of uracil to UMP at the membrane surface is very unlikely in *E. coli* B.

One aspect of uracil production in the mutant requires further explanation. The concentration level of uracil in the medium of the mutant after a 10-min period of incubation with uridine is decreased by 80% as compared to that in *E. coli* B. Since about 50% of the uracil produced in *E. coli* B is formed intracellularly, and the mutant has lost the ability to transport uridine, this accounts for a 50% reduction in uracil production in the mutant. The additional 30% decrease could be accounted for by a lower uridine phosphorylase activity in the periplasmic space of the mutant. However, shock fluid from wild-type and mutant cells show very similar activities (Table II). A possible explanation is that the activity of uridine phosphorylase in the periplasmic space of mutant cells is lower because of its association with other membrane contituents involved in uridine transport and metabolism. Disruption of such a protein complex during the shock procedure could alter its activity. Some support for such a protein complex is provided by the observation (unpublished data) that all extracellular uridine is converted to uracil and ribose 1-phosphate at extended time periods, whereas the equilibrium of the uridine phosphorylase reaction *in vitro* favors uridine formation.

Several authors have obtained evidence which has led to the suggestion that uridine transport occurs by more than one process[4,5]. The present study is consistent with this conclusion if the transport of uracil derived from uridine as well as transport of intact uridine are both considered as uridine transport. However, the existence of more than one transport process for intact uridine is open to question.

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