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Genetic and Biochemical Characterization of Distinct Transport Systems for Uracil, Uridine and Cytidine in *Salmonella typhimurium*

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Summary. 1. Uridine transport has been examined in wild type and mutant strains of *Salmonella typhimurium*. The nucleoside-specific system requires a functional uridine kinase (*udk*$^+$) as well as a periplasmic binding protein. This transport system is specific for uridine and cytidine and does not appear to contribute significantly to the uptake of deoxythymidine nor the purine nucleosides. The transport of uridine is characterized by hyperbolic kinetics with an apparent K_m for uridine of 4.3 ± 0.4 μM.

2. Uracil is transported by a completely different system which requires a functional uracil phosphoribosyl transferase (*upp*$^+$) and possesses a periplasmic binding protein.

3. Bacterial strains lacking uridine kinase (*udk*$^-$) do not transport uridine, but do transport uracil. Conversely, strains lacking uracil phosphoribosyl transferase (*upp*$^-$), transport uridine, but do not transport uracil. Thus, uridine is transported intact rather than cleaved to uracil plus the ribose moiety and subsequently transported. Furthermore, very little uridine phosphorylase (uridine + Pi → uracil + ribose-1-phosphate) is recovered in periplasmic shock fluids of *S. typhimurium*.

4. Periplasmic binding proteins were identified for both uridine and uracil. Furthermore, the uridine binding protein is separate from uridine kinase. Thus, transport of uridine and uracil require the participation of a periplasmic binding protein and the appropriate "salvage" enzyme.

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Introduction

Intracellular requirements for pyrimidine nucleotides may be satisfied by *de novo* biosynthesis, recovery of RNA degradation products, and/or exogenously supplied bases or nucleosides. The metabolism of exogenously supplied pyrimidines (bases and nucleosides) has been the subject of numerous investigations and the pathway for the utilization of cytosine, cytidine, uracil and uridine in *Salmonella typhimurium* is shown in Fig. 1 (O'Donovan and Neuhard 1970; Beck et al., 1972). In wild type *S. typhimurium*, uridine and cytidine can serve as sole carbon sources (i.e. ribose-1-P), whereas uracil and cytosine can not (Gutnick et al., 1969). Pyrimidine nucleotides can also satisfy the car-

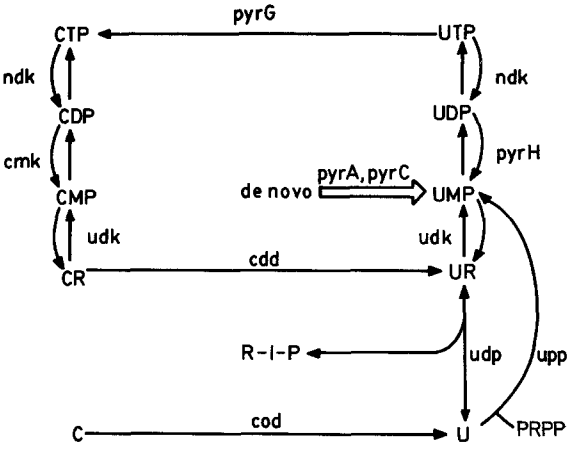

Fig. 1. Schematic representation of pyrimidine interconversion and salvage pathways with genetic loci and enzymes for *Salmonella typhimurium* LT2. *C*, cytosine; *CR*, cytidine; *U*, uracil; *UR*, uridine; *MP*, riboside 5′-monophosphate; *DP*, riboside 5′-diphosphate; *TP*, riboside 5′-triphosphate; *pyrA* specifies carbamyl phosphate synthetase; *pyrC*, dihydroorotase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase; *upp*, uracil phosphoribosyl transferase; *cdd*, cytidine deaminase; *udp*, uridine phosphorylase; *cod*, cytosine deaminase; *udk*, uridine kinase; *ndk*, nucleoside diphosphate kinase; *cmk*, CMP kinase

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bon source requirement (Yagil and Beacham, 1975). Utilizable carbon (the ribose moiety) is provided by three catabolic enzymes: a nucleotidase (nucleotide $+ H_2O \rightarrow$ nucleoside + phosphate) in *S. typhimurium*, cytidine deaminase (cytidine $+ H_2O \rightarrow$ uridine $+ NH_3$) and uridine phosphorylase (uridine + phosphate $\rightarrow$ uracil + ribose-1-phosphate). Cytidine is preferentially deaminated to uridine which is subsequently phosphorolyzed to uracil and ribose-1-phosphate (Beck et al., 1972). Furthermore, pyrimidines may be utilized as the sole source of nitrogen (Beck et al., 1972). Thus, pyrimidine nucleosides and nucleotides may serve as sole carbon source, nitrogen source, or provide intact nitrogenous base. The syntheses of nucleoside catabolizing enzymes, cytidine deaminase (encoded by *cdd*) and uridine phosphorylase (encoded by *udp*), are controlled by the cytidine regulatory gene (*cytR*), and their syntheses are induced by growth in the presence of uridine, cytidine, or adenosine. Furthermore, the synthesis of the *cyt*-enzymes appears subject to catabolic repression (Nygaard, 1973; Albrechtsen et al., 1976). In order to salvage RNA degradative products or utilize exogenous nucleotides, the nucleotides may be reutilized intracellularly or degraded to nucleosides plus inorganic phosphate and reutilized via appropriate nucleoside kinases. The salvage enzymes are required for growth only when nucleotides serve as the sole carbon source or to recover pyrimidines from degraded RNA. These processes have been the subject of extensive investigations (Hochstadt-Ozer and Stadtman, 1971 a, b, c; Hochstadt-Ozer, 1972; Hochstadt, 1974; Grenson, 1969; Von Dippe et al., 1973; Roy-Burman and Visser, 1975; and Rader and Hochstadt, 1976; Leung and Visser, 1977).

It has been proposed that uridine must be cleaved by a "vector process" in which ribose-1-phosphate is transported into the cell and uracil is released into the periplasmic space (Hochstadt, 1974). Subsequent transport of uracil (via group translocation) would be mediated by uracil phosphoribosyl transferase, resulting in the accumulation of uridine-5′-monophosphate within the cell. Appropriate membrane-bound phosphoribosyl transferase enzymes would utilize α-5-phosphoribosyl-1-pyrophosphate (PRPP) in an energy-requiring translocation (Hochstadt-Ozer and Stadtman 1971 a, b, and c; and Hochstadt-Ozer, 1972). This notion was supported by kinetic studies of purine and pyrimidine transport in whole cells (Hochstadt-Ozer and Stadtman, 1971 c) and various membrane vesicles (Hochstadt-Ozer and Stadtman, 1971 b; Hochstadt-Ozer, 1972). In fact, a direct relationship between uracil phosphoribosyl transferase associated with the membrane vesicles and uracil uptake was reported (Hochstadt, 1974). The involvement of uracil phosphoribosyl transferase in the uptake of uracil was questioned by Grenson (1969), Von Dippe et al. (1973), and Roy-Burman et al. (1975). Uracil transport in cells (Hochstadt-Ozer and Stadtman, 1971 c) and membrane vesicles (Hochstadt-Ozer, 1972; Rader and Hochstadt, 1976) was reported to be dependent on a functional UMP pyrophosphorylase (*upp*$^+$), however, uracil was reported to be transported in *E. coli* B although no uracil phosphoribosyl transferase could be measured (Von Dippe et al., 1973; Beck et al., 1972). Fast and Sköld (1977) resolved this disagreement by demonstrating that the *E. coli* enzyme required GTP activation and was present in *E. coli* B. Thus, it does appear that the *upp*$^+$ gene product *is* essential for the transport and/or retention of uracil in *E. coli*. More recently, Leung and Visser (1977) have challenged the idea that uridine uptake obligates phosphorylation to uracil and ribose-1-phosphate with subsequent, independent transport of each by group translocation (Hochstadt, 1974). These authors describe a complex inter-related series of processes involved in the transport of uracil and uridine into *E. coli*. In fact, uridine *may* be broken down to uracil + ribose-1-phosphate through a process in which only the ribose moiety is transported into the cell. Uracil does accumulate in the medium and may be subsequently transported by a base specific system. In addition, however, a separate transport system will promote the specific uptake of uridine intact. The uptake of the ribose moiety is rapid relative to the transport of either uracil or uridine and appears to be distinct. In addition, the involvement of uridine phosphorylase in the transport of the ribose moiety is uncertain. Cytidine is transported, in part, by the uridine system, but also appears to possess a second transport system which is quite distinct from the uridine/cytidine system. These conclusions are based upon transport characteristics in whole cells of mutants lacking various components involved in the transport of uracil, uridine, or cytidine. The characterization of pyrimidine nucleoside transport is not complete since the nature of the transport mutants have not been determined. The use of transport-deficient mutants in these studies has allowed the clarification of the processes involved in the transport of pyrimidine nucleotides in *E. coli*.

The studies presented in this paper identify the enzymes involved in uracil and uridine transport in *S. typhimurium* by comparing the wild type and specially constructed mutants deficient in transport and metabolic interconversions. Our studies clearly support the intact transport of uridine-cytidine in *S. typhimurium*. The transport capabilities and enzymatic activities of intact cells, the location of the enzymes and binding proteins within the cell indicate

that the transport of uracil and uridine are independent of one another and that uridine is not broken down to uracil prior to transport. In addition, cytidine is transported, in part, by the uridine transport system.

Materials and Methods

Bacterial Strains. The bacterial strains used in this study were derivatives of *Salmonella typhimurium* LT2 (listed in Table 1). We are indebted to Dr. G.A. O'Donovan who supplied strains LT2, S79, HD1043, and S177, and to Dr. J.L. Ingraham for strain JL411.

Reagents. The following radioactive chemicals were obtained from Amersham/Searle Corp; (U-^{14}C) uridine (491 mCi/mmol), (2-^{14}C) uracil (59 mCi/mmol), (2-^{14}C) cytosine (59 mCi/mmol) and from Schwarz/Mann Corp., (2-^{14}C) cytidine (40 mCi/mmol); (2-^{14}C) thymidine (54 mCi/mmol); (2-^{14}C) uridine (60 mCi/mmol); (5-^{3}H) uridine 5′-monophosphate (12.7 Ci/mmol). Unlabeled compounds were obtained from Sigma Chemical Co. All other chemicals were reagent grade and obtained commercially.

Growth Conditions. Bacterial cultures were incubated at 37° C in a Psycrotherm Incubator Shaker (New Brunswick Scientific Co., Inc). All cultures were grown overnight in Tris-ferric (TF) chloride medium, diluted and exponential growth was established (Williams and O'Donovan, 1973).

Measurement of Base and Nucleoside Transport. Cultures were harvested during exponential growth by centrifugation (2,310 × *g*) at 30° C for 15 min, washed once with sterile saline (0.85%), resuspended in phosphate buffered saline (PBS, Dulbecco A – Difco) plus 0.5% glucose, and used immediately for uptake studies. Transport mix, 0.7 M glucose in PBS Dulbecco A + 12.2 μM uridine (60 μCi/mole), was preincubated at 37° C for 5 min and transport was initiated by adding prewarmed bacterial suspension (50 μl). Samples (50 μl) were removed at 15, 30, 60, 90 and 120 s, placed on a Millipore filter (HAWP-0.45 μm) under vacuum, washed with 5.0 ml of phosphate buffered saline, and the filter was dried for 10 min on an aluminum foil covered hot plate (50–60° C). Radioactivity was determined in a Beckman LS-335 liquid scintillation counter. This procedure was repeated with the addition of sodium cyanide at 0° C to detect non-specific uptake and/or binding. These controls were always equivalent to background levels.

Preparation of Extracts. Bacterial cultures were grown to mid-log phase, harvested by centrifugation at 4° C, washed once in 40 mM potassium phosphate buffer (pH 7.2), and resuspended in the same buffer. The cells were disrupted in a French pressure cell press (American Instrument Company) at 20,000 lb/in^2, centrifuged at 2,310 × *g* at 4° C for 15 min to remove the whole cells, and the supernatant was centrifuged at 80,000 × *g* for 60 min. This supernatant and the pellet (washed and resuspended in an appropriate volume of buffer) were utilized for enzymatic assays.

Osmotic Shock Procedure. Periplasmic proteins were isolated as described by Willis et al. (1974). The osmotic fluid was concentrated by pressure filtration through an AMICON UM10 membrane DIAFLO ultrafiltration system at 4° C to a final volume of 10–25 ml. This *"periplasmic fraction"* was centrifuged at 5,900 × *g* for 10 min and assayed immediately for enzymatic activity and specific binding activity. The shocked bacteria were resuspended in 33 mM Tris-HCl (pH 7.3), crushed in a French pres-

Table 1. Bacterial strains of *Salmonella typhimurium* LT2 used in this study

Strain	Genotype	Reaction blocked	Nutritional requirements	Analog resistance
LT2	Wild type	None	None	None
S79	*pyrA81*	$NH_3 + CO_2 + ATP \rightarrow CP + ADP$	Arginine uracil	None
	udp	UR + Pi → U + R-1-P		None
S177	*upp*	U + PRPP → UMP + PPi	None	5-FU
HD1043	*pyrA81*	$NH_3 + CO_2 + ATP \rightarrow CP$	Arginine	
	pyrG	UTP → CTP	Cytidine	
	cod	C → U		5-FC
	cdd	CR → UR		5-FCdR
	upp	U + PRPP → UMP + PPi	Uridine	5-FU
	udp	UR + Pi → U + R-1-P		
JL411	*purC7*	PRAIC → PRAI-SC	Adenine	
	pyrC	CAA → DHO	Uracil	
	cod	C → U		5-FC
	cdd	CR → UR		
	udk	UR + ATP → UMP + ADP		5-FCR
				5-FUR
	udp	UR + Pi → U + R-1-P		

Abbreviations used in this Table: NH_3, ammonium; CO_2, carbon dioxide; ATP, 5′-adenosine triphosphate; CP, carbamyl phosphate; UR, uridine; Pi, inorganic phosphate; U, uracil; R-1-P, ribose-1-phosphate; U, uracil; PRPP, 5-phosphoribosyl-1-pyrophosphate; UMP, 5′-uridine monophosphate; PPi, inorganic pyrophosphate; UTP, 5′-uridine triphosphate; CTP, 5′-cytidine triphosphate; PRAIC, 1-(5′phosphoribosyl-5-aminoimidizole-4-carboxylate; PRAI-SC, 1-(5′-phosphoribosyl)-4-(N-succinocarboxamido)-5-aminoimidizole; CAA, carbamyl aspartate; DHO, dihydrorotate; 5-FU, 5-fluorouracil; 5-FC, 5-fluorocytosine; 5-FCdR, 5-fluorodeoxycytidine; 5-FCR, 5-fluorocytidine; 5-FUR, 5-fluorouridine

sure cell, centrifuged at $5,900 \times g$ for 10 min, and the pellet was discarded. The remaining supernatant was centrifuged at $80,000 \times g$ for 60 min. The supernatant was designated the *"cytosol fraction"*. The pellet was washed twice with 33 mM Tris-HCl (pH 7.3) resuspended in the same buffer and henceforth is designated the *"membrane fraction"*. The membrane fraction represents the cell envelope and was not fractioned further.

Transport capability of the intact bacterial cells was analyzed before and after osmotic shock, and all fractions (periplasmic, cytosol, and membrane) were assayed for enzyme and binding activity after disruption.

Binding Activity Studies. The various fractions were analyzed for binding activity by equilibrium dialysis in a multichamber apparatus described by Furlong et al. (1972). All fractions were assayed in triplicate at two concentrations of protein. Dialysis was carried out at 4° C for 36–48 h, at which time control equilibrium had been established. Nonspecific binding activity was tested with fractions that had been heated in a boiling water bath for 30 min. Samples (50 µl) were withdrawn from each side of the chamber after dialysis. added to 7.5 ml of Aquasol (New England Nuclear), and counted as described above.

Enzyme Assays. All assays were carried out at pH 7.4 and 37° C, using radiochemical techniques employing labeled (2-^{14}C) substrates. The reaction products were resolved by either thin-layer (Eastman Chromagram cellulose precoated plastic sheets) or diethylaminoethyl (DE 81 disks; Whatman) chromatography. Specific activities of the enzymes (nanomoles product per min per mg of protein) were determined under conditions in which product formation was proportional to amount of cell extract added and time.

Uracil phosphoribosyl transferase (UMP pyrophosphate phosphoribosyl transferase, EC 2.4.2.9) was assayed as previously described (Flaks, 1963) with a slight modification. The incubation mixture had a volume of 200 µl and the following composition: potassium phosphate buffer, 50 mM; $MgCl_2$, 5 mM; reduced glutathione, 5 mM; 5-phosphoribosyl-1-pyrophosphate $(PRPP)^-$ Na salt, 0.3 mM; (2-^{14}C) uracil, 0.048 mM (59 µCi/µmole); and appropriate cell-free extract. Enzymatic activity was monitored by withdrawing 30 µl samples at 1, 2 and 3 min intervals. The samples were applied directly on dry DE 81 disks, washed on a Millipore filtration manifold with 30 ml of 1 mM ammonium formate followed by 2.0 ml of 95% ethanol, and placed in scintillation vials. The disks were dried at 45° C for 30 min and radioactivity was determined.

Uridine phosphorylase (uridine orthophosphate ribosyltranferase, EC 2.4.2.3) was assayed by monitoring the phosphorolysis of (2-^{14}C) uridine to (2-^{14}C) uracil. The incubation mixture had a volume of 200 µl and the following composition: potassium phosphate buffer, 50 mM; (2-^{14}C) uridine, 2.004 mM (0.49 µCi/µmole); and the appropriate dilution of cell-free extract. Enzymatic activity was monitored by withdrawing 30 µl samples at 5 min intervals. The samples were applied directly on cellulose coated plastic plates at 65° C (hot-air blower). The product of the reaction (uracil) was separated form the substrate (uridine) chromatographically in one dimenion using n-Butanol-Water (86:14, v/v) as the solvent system, in rectangular glass tanks. Uracil and uridine were located under ultraviolet light, marked with a soft lead pencil, excised with scissors, and the radioactivity was determined as described above.

Uridine kinase (adenosine 5′-triphosphate (ATP); uridine 5-phosphotransferase, EC 2.7.1.48) was assayed by monitoring the phosphorylation of uridine to uridine-5′-monophosphate. The incubation mixture has a volume of 200 µl and the composition: Tris-HCl, 50 mM; ATP (disodium salt), 5 mM; $MgCl_2$, 5 mM; β-mercaptoethanol, 2.5 mM; (2-^{14}C) uridine, 1.019 mM (0.98 µCi/µmole); and appropriate cell-free extract. Termination of the enzymatic reaction, separation of the product (uridine-5′-monophosphate) and counting were performed as described for uracil phosphoribosyl transferase.

Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) was assayed by monitoring the deamination of (2-^{14}C) cytosine to (2-^{14}C) uracil. The incubation mixture had a volume of 200 µl and the following composition: potassium phosphate, 50 mM; (2-^{14}C) cytosine, 2.0 mM (0.49 µCi/µmole); and appropriate cell-free extract. Enzymatic activity and termination of the reaction were as described above for uridine phosphorylase. Chromatographic separation of cytosine from uracil was carried out in one dimension with cellulose plates using methanol HCl-H_2O (70:20:10). Location of the spots, excision, and scintillation counting were as described above.

Cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) was assayed by monitoring the deamination of (2-^{14}C) cytidine to (2-^{14}C) uridine. The incubation mixture has a volume of 200 µl and the following composition: potassium buffer, 50 mM; (2-^{14}C) cytidine, 4.0 mM (0.25 µCi/µmole); and appropriate cell-free extract. Enzymatic activity and termination of the reaction were as described above for uridine phosphorylase. Chromatographic separation of uridine from cytidine was carried out in one dimension on cellulose plates using methanol HCl-H_2O (70:20:10). Location of the spots, excision, and scintillation counting were as described above.

Cytidine 5′-triphosphate synthetase (uridine 5′-triphosphate ammonia ligase (ADP), EC 6.3.4.2) was assayed as previously described (Williams et al., 1978).

Protein determination on the extracts were carried out by the method of Lowry (Lowry et al., 1951), with bovine serum albumin as the standard.

Results

1. Verification of Relevant Genotypes. The *S. typhimurium* strains listed in Table 1 were used as a means of analyzing the transport functions of enzymes or binding proteins in the periplasmic space, cytosol, and/or the membrane(s) and thus it was important to verify the genotypes by enzyme assay. The verification of the nutritional requirements and analogue resistance of the strains utilized in this study are outlined in Table 1. When the enzymatic activities of these strains were determined, it became apparent that strains contain appropriate enzymatic blocks (Table 2). Uridine phosphoribosyl transferase (URPTase) was undetectable in strains S177 and HD1043, whereas LT2, JL411, and S79 exhibited specific activities of $30–40 \times 10^{-2}$ nmoles min^{-1} mg $protein^{-1}$. No uridine kinase (URKase) activity could be detected in strain JL411, while the other four strains clearly possessed that enzymatic activity. Uridine phosphorylase (URPase) was detectable in purported *udp*$^-$ strains, however, S79 possessed less than 7 per cent of the wild type strain activity and HD1043 had less than 20 per cent of the wild type levels. The salvage of cytosine and cytidine is carried out by cytosine deaminase (encoded by *cod*) and cytidine deaminase (encoded by *cdd*). The activities of these two enzymes

Table 2. Characterization of the *Salmonella typhimurium* LT2 strains used in this study

Strain	Genotype	Specific activity					
		URPTase[a] (*upp*)	URKase[a] (*udk*)	URPase[b] (*udp*)	CRDase[b] (*cdd*)	CDase[b] (*cod*)	CTPSase[b] (*pyrG*)
LT2	Wild type	40	170	42.3	38.9	20.7	120
S177	*upp*	UD[c]	70	54.1	32.2	25.9	–[d]
S79	*pyrA, udp*	32	40	3.3	45.7	14.4	–
HD1043	*pyrA, pyrG cdd, cod, upp, udp*	UD	30	11.6 –	0.4 –	0.4 –	UD
JL411	*purC7, cdd-9, udp-8, cod-8, udk-6, pyrC1502*	40	UD	UD	UD	UD	–

[a] Specific activity = nmoles min^{-1} mg $protein^{-1} \times 10^{-1}$
[b] Specific activity = nmoles min^{-1} mg $protein^{-1}$
[c] UD = undetected
[d] – = Not performed

Table 3. Transport of (2-^{14}C) pyrimidines in various strains of *S. typhimurium* LT2

Strain[b]	Transport Activity[a]			
	uracil	uridine	cytidine	thymidine
LT2	100	100	100	100
S177 (*upp*)	8	120	108	108
S79 (*udp*)	120	95	90	105
HD1043 (*upp, udp*)	5	102	103	101
JL411 (*udp, udk*)	89	4	58	93

[a] Transport was determined as described in the text with 12 μM pyrimidines and is expressed as percentage of the transport of the wild type strain LT2. Transport is represented as relative specific activities (pmoles min^{-1} mg $protein^{-1}$)
[b] See Table 2 for detailed genotypic characterization of strains. All strains were assayed at mid-log growth in TF medium + uridine + adenine (uracil was substituted for uridine in the growth of JL411)

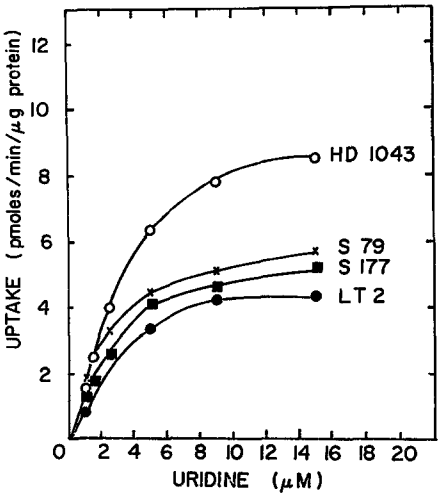

Fig. 2. Uridine uptake in strains of *Salmonella typhimurium*. Uptake was measured under standard assay conditions while varying the uridine concentration. (●) LT2 is representative of the wild-type, and takes up uracil and uridine. (○) HD1043 is *pyrA*$^-$, *pyrG*$^-$, *upp*$^-$, *cdd*$^-$, *udp*$^-$, *cod*$^-$ and did not take up uracil. (■) S177 is *upp*$^-$ and did not take up uracil. (×) S79 is *pyrA81*$^-$, *udp*$^-$, and takes up both uracil and uridine

were greatly reduced in HD1043 and JL411. Finally, the formation of CTP from UTP via cytidine-5′-triphosphate synthetase was blocked in HD1043. Thus, the mutants had the correct blocks for evaluation of pyrimidine transport systems.

2. Transport of Uracil. Strains LT2, JL411 and S79 had functional uracil phosphoribosyl transferase activity (specified by *upp*$^+$) and transported uracil. Strains S177 and HD1043 lacked the functional enzyme and did not transport uracil. Thus, uracil phosphoribosyl transferase activity is required to transport uracil (Table 3).

Uridine phosphorylase activity does not appear to be involved in uracil transport since strain S79 has less than 8% of the enzymatic activity of the wild-type strain and still has transport capability comparable to that strain. Thus, the *upp*$^+$ but not the *udp*$^+$ gene product is required for uracil transport.

3. Transport of Uridine. Uridine kinase (specified by *udk*$^+$) is essential for uridine transport (Fig. 2). JL411 (*udk*$^-$) transported uridine at a rate only 5% of the wild type strain (Fig. 3A). Nonetheless, JL411 demonstrated uridine binding activity comparable to

Table 4. Kinetic constants for uptake of uridine by *Salmonella typhimurium* strains LT2, S177, S79 and HD1043

Strain	Kinetic constants[a]		
	K_m	V_{max}	n[b]
LT2	4.28±0.37 (100)	5.35±0.18 (100)	1.03
S177	3.54±0.34 (83)	6.41±0.19 (120)	1.00
S79	2.14±0.26 (50)	5.13±0.12 (96)	0.96
HD1043	3.70±0.66 (86)	9.83±0.56 (183)	1.00
JL411	[c]		

[a] Kinetic studies were carried out with [2-^{14}C] uridine and [U-^{14}C] uridine K_m=μM. V_{max}=pmoles min^{-1} μg protein^{-1}

[b] n=Hill coefficient of cooperativity

[c] No uptake was consistently observed in JL411

the wild-type strain (Fig. 3B). Thus, a periplasmic binding protein for uridine, independent of the uridine kinase, may be involved in the transport process. Other mutations affecting pyrimidine salvage enzymes (*upp*$^-$, *udp*$^-$, *cdd*$^-$, *cod*$^-$) did not affect uridine uptake; i.e. uptake occurred in all cases (Table 3).

4. Transport of Cytidine. Cytidine (2-^{14}C) is transported by all of the strains used in these studies, including JL411, although transport in that *udk*$^-$ strain is reduced relative to the other strains (Table 3).

5. Transport of UMP. Strain HD1043 cannot transport uracil but it has retained uridine transport capability (Fig. 2). This strain also takes up radioactivity from UMP although nucleotides *per se* are not transported (Fig. 4; Yagil and Beacham, 1975). This accumulation of internal radioactivity is brought about by the periplastic phosphorolysis of UMP to uridine and the subsequent transport of radioactive uridine (O'Donovan and Neuhard, 1970). Similar uptake was observed in LT2 and S177.

The breakdown of UMP to uridine by 5′-nucleotidase or non-specific phosphatase leads to subsequent uptake. This provides a route for the utilization of exogenously supplied UMP as a pyrimidine source.

6. Effects of Bases, Nucleosides, and Ribose Phosphates on Transport. The competitive effect of bases and nucleosides on the transport of uridine was compared in strain LT2 (Table 5). Uridine, cytidine, deoxyuridine, and deoxycytidine were inhibitors of uridine transport, whereas, uracil, and cytosine were not (likewise, all other bases has no effect). Uridine and cytidine were effective inhibitors of uridine transport, deoxythymidine was not as effective. In reciprocal experiments, uridine did not affect deoxythymidine transport, but it did inhibit cytidine transport by 38% when concentrations of both nucleosides were 12 μM. This specificity extends to nucleoside analogues (e.g. 6-azauridine inhibits uridine transport while 3-deazauridine does not). Ribose-1-phosphate did not inhibit uridine uptake and ribose-5-phosphate was only 15% inhibitory in wild-type strain (data not shown).

Table 5. The effect of pyrimidines on (2-^{14}C) uridine uptake by *Salmonella typhimurium* LT2

Compound	Concentration (μM)	Per cent inhibition of uridine uptake
uridine[b]	12	60
	24	71
	122	90
cytidine[b]	12	58
	24	76
	122	90
thymidine	12	21
	24	20
	122	26
uracil	12	7
	24	3
	122	9
cytosine	12	4
	24	9
	122	6

[a] The concentration of (2-^{14}C) uridine was 12 μM

[b] Deoxyribosides were equally effective inhibitors as the ribonucleosides

7. Effect of Metabolic Inhibitor on Uridine Transport. Sodium cyanide was the most effective metabolic inhibitor of uridine transport, while sodium azide (another respiratory inhibitor) inhibits 50% at 500 μg/ml. In addition, 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation, reduced transport to 7% of the control rate.

8. Separation of Uridine/Cytidine Transport from Uracil Transport. Several lines of evidence indicate that base and nucleoside transport are accomplished by separate processes. The kinetic constants for the transport or uridine (2-^{14}C) or (U-^{14}C), by *S. typhimurium* strains LT2, S177, S79, and HD1043 are compared in Table 4. The reciprocal plots of transport velocities *versus* concentration are linear and suggest that transport of uridine is *mediated* by one system (data not shown). The apparent Kms for all strains are similar, even for those that are unable to transport uracil. The apparent V_{max} for strain HD1043 is higher than the other strains. Strain HD1043 does not transport uracil, had no uracil phosphoribosyl transferase activity, and has a reduced uridine phosphorylase activity. This observation suggests that the ability or inability to degrade uridine and transport uracil does not affect uridine transport. The Hill coefficient of cooperativity (n) is not significantly different from

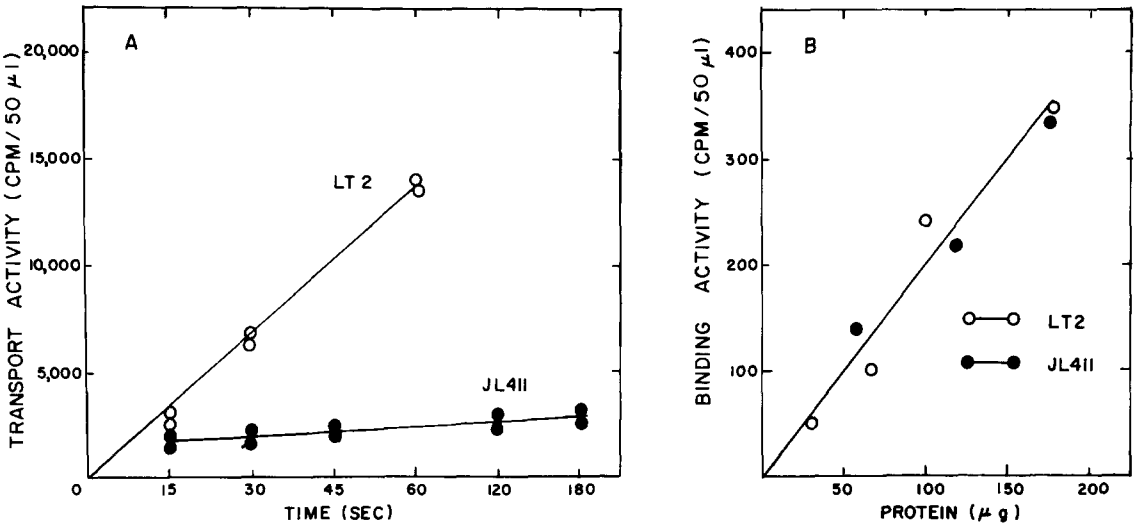

Fig. 3. Uridine uptake and binding activity of *Salmonella typhimurium* strains LT2 and JL411. Uptake and binding activity was measured in the presence of 10 μM uridine under standard assay conditions using [2-^{14}C] uridine. Uptake activity A, and binding activity B; (o), LT2 and ( ), JL411 (*purC7*$^-$, *cdd9*$^-$, *udp8*$^-$, *cod8*$^-$, *udk6*$^-$, *pyrC1502*$^-$)

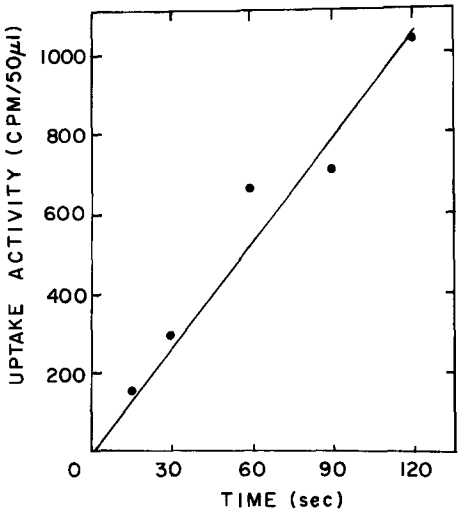

Fig. 4. Uptake activity in the presence of [^{3}H] UMP by *Salmonella typhimurium* strain HD1043. Uptake activity was measured in the presence of 10 μM uridine 5′-monophosphate under the standard assay conditions using [^{3}H] UMP which was 99% [^{3}H] UMP. The observed uptake could not be accounted for by contaminating uridine or other compounds

1.0 for any of these strains which indicates a lack of both positive or negative cooperativity and perhaps indicate a single transport system. The kinetic data were compared using a program (R.C. Jackson, personal communication) which fits parameters to transport data in which facilitated and passive transport occur simultaneously:

$$v = \frac{V_{max} \times S}{S + K_m} + k[S]$$

where "k" is a first-order rate constant for passive diffusion (Hoare, 1972). In this analysis no passive transport (i.e., $k=0$) was observed. This evidence further indicates that a single kinetic system is involved in the uptake of uridine. No kinetic differences in uridine uptake were observed whether the base and the ribose moiety (U-^{14}C) or just the base (2-^{14}C) were used in the transport studies. Thus, uridine appears to be transported into *S. typhimurium* intact by a single process.

9. Induction of Uridine Transport. The transport of uridine appears subject to induction by growth in uridine-containing media. Strains grown in the presence and absence of uridine increase in specific transport capacity (up to 250% in HD1043). Further studies on the adaptation of strain LT2 to growth on uridine as a sole source of carbon indicated that uridine was most efficient in inducing its own transport (J.C. Williams, unpublished observation).

10. Compartmentation of Enzymatic and Binding Protein Functions. A clear understanding of transport cannot be developed without localization of contributing enzymes and binding proteins. Osmotic shock release of periplasmic enzymes and binding activities decreased the transport of uracil and uridine by 44 and 18%, respectively. The periplasmic fraction fluid contained uracil phosphoribosyl transferase (16%, involved in uracil transport), uridine phosphorylase (1.1%), uridine kinase (17%, involved in uridine transport), cytosine deaminase (4%), and cytidine deaminase (3.4%). The majority of the enzyme activities were recovered in the cytoplasmic fraction (Ta-

Table 6. Localization of the enzymes of pyrimidine salvage in shocked *Salmonella typhimurium* LT2

Enzyme	Compartmental enzymatic activities[b,c]		
	Periplasm	Membrane	Cytosol
Uracil[a]	0.15 (16)	0	0.77 (84)
Uridine phosphorylase	9.22 (1.1)	1.45 (0.2)	808.00 (98.7)
Uridine kinase	2.62 (17)	0.24 (2)	12.29 (81)
Cytosine deaminase	2.63 (4)	0.31 (0.5)	61.80 (95.5)
Cytidine deaminase	20.50 (3.4)	1.10 (0.2)	574.00 (96.4)

[a] Uracil phosphoribosyl transferase

[b] The periplasmic fraction was prepared by concentrating extracellular fluids following osmotic shock as described in the text. The membrane and cytosol fraction was isolated following explosive disruption in a French Pressure Cell (see text)

[c] Specific activities = nmoles min^{-1} mg $protein^{-1}$. Percentage of total activity in parentheses

Table 7. Comparison between glucose, arginine, uridine, and uracil binding activities[a]

Substrate	Compartmental Binding Activity[b]		
	Periplasm	Membrane(s)	Cytoplasm
Glucose	0.43 (97)[c]	0.01 (3)	0
Arginine	0.67 (97)	0.01 (1.5)	0.01 (1.5)
Uridine	0.50 (100)	0	0
Uracil	0.10 (100)	0	0

[a] Binding activity recovered from *S. typhimurium* LT2

[b] Compartmentation described in Table 6

[c] Specific activity = nmoles bound/mg protein. Percentage of the total binding activity in parentheses

ble 6). The large proportion of activity in the cytoplasm indicates that these enzymes are required for proper utilization of endogenous uridine and uracil. Very little enzymatic activity was observed in the membrane fraction. More importantly, both uridine and uracil binding activities were localized in the "periplasmic fraction" (Table 7). These binding proteins were labile and adhered to nitrocellulose membrane filters. The binding proteins for glucose and arginine were monitored as controls since these proteins have been shown to be released from the periplasm (Willis et al., 1974; Furlong et al., 1972).

Discussion

Uridine is transported into *S. typhimurium* intact without phosphorolysis to uracil + ribose-1-phosphate. Uridine kinase: (uridine + ATP → UMP + Pi) is an essential component of uridine transport and neither uridine phosphorylase (uridine + Pi → ribose-1-phosphate + uracil) nor uracil phosphoribosyl transferase (uracil + 5-phosphoribosyl-1-pyrophosphate → UMP + inorganic pyrophosphate) is required. Furthermore, uridine kinase is not the only protein involved in uridine transport since a specific uridine-binding protein is present in uridine kinase deficient strains such as JL411 (*udk*$^-$).

Nucleoside transport studies in whole cells of *E. coli* have revealed that the nucleoside catabolizing enzymes are found within the cell membrane and that extracellular degradation occurs within a few seconds in the periplasm. In these cells, catabolism is so rapid that transport must be examined in mutants incapable of catabolism or in the presence of catabolic inhibitors (Leung and Visser, 1977). Such extensive breakdown does not occur in *S. typhimurium* and uridine transport may be examined directly. Exogenous catabolism of uridine is possible via uridine phosphorylase in *Salmonella* but this is insignificant in cells capable of uridine transport. The transport of intact uridine is clearly demonstrated by (i) the inability of JL411 (*udk*$^-$) to transport (2-^{14}C) uridine or (U-^{14}C) uridine although (2-^{14}C) uracil is transported, (ii) the inability of HD1043 (*udp*$^-$ *upp*$^-$) and S177 (*upp*$^-$) to transport (2-^{14}C) uracil although (2-^{14}C) uridine and (U-^{14}C) uridine are transported, (iii) the kinetics of uridine transport are similar in several mutants independent of the strains ability to transport uracil or degrade uridine.

A clear distinction has been made between uridine transport activity (uridine kinase) and a uridine binding protein. Binding proteins for both uracil and uridine were located in the osmotic shock fluids (periplasm) of strain LT2. Strain JL411 continues to bind uridine even though it is *udk*$^-$ and cannot transport uridine. This binding activity appears to be distinct from uridine kinase since uridine was transported in the presence of 3-deazauridine, an analogue that inhibits uridine kinase activity competitively in vitro. Other uridine analogues (e.g. 6-azauridine) inhibit both transport and enzymatic activity (unpublished observation).

The nucleoside transport system does not discriminate between cytidine and uridine, and it is only partially inhibited by deoxythymidine. Deoxythymidine does not appear to be transported by this system since no significant reduction in deoxythymidine transport is observed in *udk*$^-$ mutants incapable of uridine transport. By this argument, cytidine is also transported by another system in *S. typhimurium*, since cytidine transport is observed in the absence of uridine transport (*udk*$^-$ strains). Leung and Visser (1977) recently demonstrated the transport of intact uridine and cytidine in *E. coli* is accomplished by

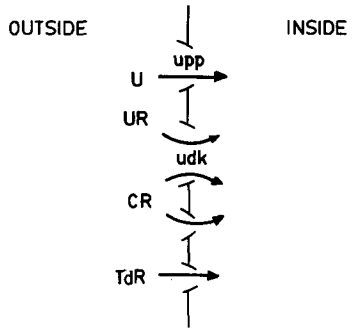

Fig. 5. Schematic representation of uracil, uridine, cytidine and deoxythymidine transport system. (See the legend for Fig. 1 for explanation of abbreviation)

two independent systems similar to those observed in these studies.

Our results suggest that the uridine transport function of uridine kinase is tightly associated with the membrane whereas uracil phosphoribosyl transferase possesses a weaker association. Surpisingly, uridine phosphorylase was found in the cytoplasm extensively where its function may be to provide ribose-1-phosphate to the cell after transport of intact nucleoside. The specificity of uridine/cytidine transport of *S. typhimurium* is a remarkable departure from the two general transport systems for nucleosides reported for *E. coli* K-12 (Munch-Petersen and Mygind, 1976). In this bacterium, one system transports all pyrimidine nucleosides and adenosine but not guanosine. The other transport system transports all nucleotides. These two systems have been separated in *E. coli* K-12 through the isolation of mutants resistant to showdomycin, a uridine analogue; the resistant strains possess the system which transports all nucleosides. All strains of *S. typhimurium* used in these studies possess similar sensitivities to showdomycin even though the ability to transport uridine and uracil varies significantly from strain to strain. It is possible to select for showdomycin resistance in *S. typhimurium* but the transport characteristics of these resistant mutants have not been examined extensively.

Uridine/cytidine transport is apparently under the control of induction by growth in cytidine or uridine. Since the catabolic enzymes involved in nucleoside salvage/reutilization are under the control of *cytR* (Nygaard, 1973; Krajewska and Shugar, 1975). The uridine transport mechanism may be closely linked to this regulatory system. The question arises as to whether these inductive mechanisms might also induce the number of binding proteins, enzymes involved in transport, or protein involved in the regulation of *de novo* pyrimidine biosynthesis. We propose the following scheme for the transport of uracil, uridine, cytidine, and deoxythymidine (Fig. 5). In this model uridine is transported by a single system involving uridine kinase (*udk*); cytidine is transported by the uridine system and a second, uncharacterized system. Uracil is transported independently of the ribonucleosides and requires uracil phosphoribosyl transferase (*upp*). The transport of deoxythymidine has not been characterized but appears separate from these systems. The transport model suggests that a new class of transport deficient mutants can be identified as lacking uridine or uracil binding activity while retaining the respective enzyme activities and, in fact, Beck et al. (1972) identified a genetic locus (*udc*, now designated *udh*, J.L. Ingraham, personal communication) which does not transport uridine and confers resistance to 5-fluorouridine. This mutation is nonallelic to *udp* and *udk*; *udh* may encode the specific binding protein(s).

It is clear that uridine can be transported intact without degradation to uracil plus ribose-1-phosphate. It might be possible for uridine to be transported without phosphorylation to UMP, although it might not be retained within the cell unless converted to UMP. If this is the case, then the process of group transloction would not be involved in base and nucleoside transport.

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