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Regulation of Purine Utilization in Bacteria

VII. Involvement of Membrane-Associated Nucleoside Phosphorylases in the Uptake and the Base-Mediated Loss of the Ribose Moiety of Nucleosides by *Salmonella typhimurium* Membrane Vesicles

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Although uridine and adenosine are converted by membrane-associated nucleoside phosphorylases to ribose-1-phosphate (ribose-1-P) and the corresponding bases (uracil and adenine), only ribose-1-P is accumulated within *Salmonella typhimurium* LT2 membrane vesicles. In accordance with these observations, no uptake is observed when the vesicles are incubated with the bases or nucleosides labeled in their base moieties. The vesicles lack a transport system for ribose-1-P, since excess ribose-1-P does not inhibit the uptake of the ribose moiety of uridine. In addition, there is no exchange with preaccumulated ribose-1-P. Thus, uridine, rather than ribose-1-P, must serve as the initially transported substrate. The uptake of the ribose portion of uridine is coupled to electron transport, and the levels to which ribose-1-P are accumulated may be reduced by adding various bases to the reaction mixtures. The bases appear to inhibit the uridine phosphorylase reaction and/or cause an efflux of ribose-1-P from the vesicles. This loss of ribose-1-P reflects the accumulation of nucleosides in the external medium after being synthesized within the membranes. Synthesis of the nucleosides from intravesicular ribose-1-P and exogenous bases proceeds even though the bases are not accumulated by the vesicles. Furthermore, ribose-1-P cannot significantly inhibit uridine phosphorylase activity unless the membranes are disrupted. These observations indicate that the membrane-associated nucleoside phosphorylases may have a transmembranal orientation with their base and ribose-1-P binding sites on opposite sides of the membranes. Such an asymmetric arrangement of these enzymes may facilitate the uptake of the ribosyl moiety of nucleosides by a group translocation mechanism. Thus, nucleosides may be cleaved during the membrane transport process, with the resultant bases delivered to the external environment while ribose-1-P is shunted to the intravesicular space.

The utilization of exogenous nucleic acid precursors depends on the presence of salvage enzyme pathways (1, 8). Previous work from this laboratory has shown that purine nucleosides must be cleaved into free bases prior to uptake (2), and this observation has been recently confirmed (23). Subsequent transport of these bases is mediated by membrane-associated phosphoribosyltransferases that convert the bases into their nucleoside monophosphates as they pass through the membranes (2, 6, 7, 10). Thus, the uptake of purine bases by *Escherichia coli* and *Salmonella typhimurium* involves a group translocation whereby transport and initial metabolism occur simultaneously. Since the phosphoribosyltransferases for adenine and uracil are not detected under the present experimental conditions, we have been able to extend the previous studies by following the fate of the ribose moiety of nucleosides only.

(A preliminary report of this investigation was presented at the 75th Annual Meeting of the American Society for Microbiology, New York, 27 April-2 May, 1975.)

MATERIALS AND METHODS

Materials. All chemicals and reagents were of the highest quality available from standard commercial sources. Radiochemicals were obtained from Amersham-Searle Corp. Inosine phosphorylase, which was purchased from Boehringer Mannheim Corp., was reacted with [U-^{14}C]inosine (277 mCi/mmol) to produce hypoxanthine and ribose-1-phosphate (ribose-1-P). The radiolabeled ribose-1-P was isolated

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for use as a chromatographic standard by applying the entire reaction mixture to a column of Dowex-2 and eluting with increasing concentrations of potassium phosphate buffer (pH 7.5).

Bacteria and cultural conditions. *S. typhimurium* strain LT2 was grown aerobically in the minimal salts medium of Mikulka et al. (16) by shaking on a rotatory shaker at 37°C. The medium was supplemented, per liter, with: $FeSO_4 \cdot 7H_2O$, 0.5 mg; uridine, 25 mg; and succinic acid, 5 g. In one experiment 0.1% glycerol was substituted for succinate, as indicated in Results. Cells were harvested in the late-log phase of growth.

Preparation of membrane vesicles. We have found that the ethylenediaminetetraacetate-lysozyme procedure described by Kaback (12) is not very efficient for producing membrane vesicles from *S. thyphimurium*. Therefore, most of the vesicles used in the present experiments were prepared by a slight modification of the techniques developed by Osborn et al. (18). The complete procedure is described elsewhere (4), with the exception that $MgCl_2$ was added at a final concentration of 10 mM after lysis of the spheroplasts. The vesicles were frozen in liquid nitrogen and stored at −70°C in 0.1 M potassium phosphate buffer, pH 7.0.

Protein determination. Protein was assayed by the method of Lowry et al. (15), with bovine serum albumin as standard.

Transport assays. Uptake assays were performed at 30°C in a total volume of 100 μl, having final concentrations of 50 mM potassium phosphate buffer, pH 7.0, and 10 mM $MgSO_4$. Vesicle protein concentration varied between 13 and 85 μg/100 μl. Transport assays were initiated by the introduction of radioactive substrate 15 s after the addition, where required, of an energy source. Usually potassium succinate, pH 7.0, was used at a final concentration of 50 mM. The specific activities and final concentrations of the radiolabeled nucleic acid precursors were as follows: [U-^{14}C]uridine (509 mCi/mmol), 10 μM; [2-^{14}C]uridine (57 mCi/mmol), 88 μM; [2-^{14}C]uracil (60 mCi/mmol), 200 μM; [U-^{14}C]-adenosine (585 mCi/mmol), 9 μM; and [8-^{14}C]adenosine (47 mCi/mmol), 106 μM. The reactions were terminated at various times by adding 2 ml of 0.5 M NaCl, and the suspensions were immediately filtered through 0.45-μm nitrocellulose filters (Millipore Corp., Bedford, Mass.). The test tubes and filters were then washed twice with 2-ml volumes of 0.5 M NaCl. Zero-time controls were diluted with 2 ml of 0.5 M NaCl prior to addition of radioactive substrates and were immediately filtered. Inhibitors were preincubated with membrane vesicles for 15 min before initiating the reactions. When testing the effect of exogenous substrates on transport, the various bases and sugars were added at a final concentration of 1.25 mM. After the addition of succinate, the reactions were started with the introduction of radioactive uridine at a concentration of 10 μM.

Chromatographic analysis. After filtration of the reaction mixtures, the filters were dried, cut into small pieces, and extracted twice at 60°C for 5 min with 1-ml portions of distilled water. The eluates were combined and then lyophilized. After addition of 50 to 100 μl of distilled water, 5 μl of each reaction mixture was applied to cellulose or polyethyleneimine-cellulose thin-layer plates (Eastman Kodak). These plates were developed in solvent 2, solvent 9 [water-saturated $(NH_4)_2SO_4$-0.1 M phosphate buffer, pH 6.0-isopropanol; 79:19:2], or solvent 27 (isobutyric acid-1 N NH_4OH-0.2 M ethylenediaminetetraacetate; 100:60:0.8) (5). Radioactive standards of various sugar or nucleic acid precursors were also applied to the plates.

Autoradiography and radioactive counting. The chromatograms were subjected to autoradiography by exposure to Kodak medical X-ray film (no screen). Filters onto which the membrane vesicles had been collected were counted either in a Nuclear-Chicago gas-flow counter or a Beckman liquid scintillation spectrophotometer. Spots on the chromatograms that corresponded to darkened areas on the autoradiographs were cut out and placed in liquid scintillation vials with appropriate scintillation fluid (5).

Efflux of preloaded ribose-1-P. After 15 min of incubation with [U-^{14}C]uridine to preload membrane vesicles with ribose-1-P, 100-μl portions of the transport reaction mixture (total volume, 1.5 ml) were distributed to several tubes. These tubes contained potassium phosphate, $MgSO_4$, and a nucleic acid base or sugar. In effect, the original uridine substrate was diluted fivefold while the vesicles were being exposed to extravesicular bases and sugars at concentrations of 2.5 mM. The reactions were terminated at intervals by dilution with 0.5 M NaCl and subsequent filtration.

Enzyme assays. The effect of various bases and sugars on the membrane-associated uridine phosphorylase reaction was determined by their addition at a final concentration of 1.25 mM. In some experiments Triton X-100 was added at 0.1% (vol/vol), which led to a clearing of the turbid membrane suspensions. When measuring any phosphorylase activity, succinate was omitted and the following substrates were present: [U-^{14}C]uridine (509 mCi/mmol), 20 μM; [U-^{14}C]adenosine (585 mCi/mmol), 17 μM; and [U-^{14}C]inosine (538 mCi/mmol), 19 μM. The enzyme assays were terminated at various intervals by boiling for 5 min. A zero-time control consisted of a preboiled sample to which radioactive nucleoside was subsequently added. Portions (5 μl) of the reaction mixtures were applied directly without prior filtration to thin-layer plates for chromatographic analyses.

Effect of Triton X-100 on uridine phosphorylase activity. In these experiments the enzyme assays contained 1.25 mM [U-^{14}C]adenine (281 mCi/mmol), 10 μM unlabeled uridine, and, when necessary, 2.5 mM unlabeled ribose-1-P. To determine the amount of radioactive adenosine produced, 5 μl of the reaction mixtures was applied to cellulose plates. These were chromatographed in two directions. After 1 M ammonium acetate, pH 7.0, was used in the first direction, the thin-layer plates were chromatographed with 85% saturated ammonium bicarbonate (16 g/100 ml). The adenosine spots were then counted in a liquid scintillation spectrophotometer.

RESULTS

Uptake of radioactivity derived from radiolabeled uridine. The time course for the uptake of radioactivity from uridine and uracil into *S. typhimurium* membrane vesicles is presented in Fig. 1. The initial rate of intravesicular accumulation, as well as the steady-state levels of radioactivity derived from uniformly labeled uridine, are stimulated by the presence of an energy source such as succinic acid. The dotted line in Fig. 1 represents the expected uptake for [2-^{14}C]uridine based on the levels of radioactivity from [U-^{14}C]uridine taken up in the presence of succinate. However, no radioactivity is found within vesicles incubated for 15 min with uridine specifically labeled in the base moiety. The vesicles also fail to take up [2-^{14}C]uracil. These results suggest that the vesicles retain the ribose portion of uniformly labeled uridine.

Identification of intravesicular contents. To verify that the sugar moiety of the uridine molecule is accumulated by membrane vesicles and to identify the exact nature of the ribose compound(s), vesicle contents were extracted with distilled water at 60°C and then analyzed by thin-layer chromatography with several solvent systems. Only one major radioactive compound appears in the chromatograms developed in solvents 2 and 9 (Fig. 2 and 3, respectively). It is attributable to either ribose-1-P or ribose-5-phosphate (ribose-5-P). To differentiate between these two sugars, the vesicle contents were applied to polyethyleneimine thin layers and chromatographed in solvent 27. The predominant intravesicular compound was found

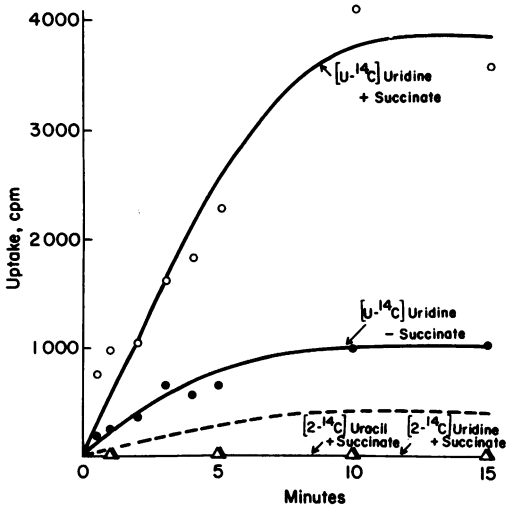

FIG. 1. *Rate of uptake of radioactivity from uridine and uracil in the presence and absence of succinic acid. Reaction mixtures (100 μl) contained 50 mM potassium phosphate (pH 7.0), 10 mM magnesium sulfate, 13.4 μg of membrane vesicle protein, and either 10 μM [U-^{14}C]uridine (509 mCi/mmol), 88 μM [2-^{14}C]uridine (57 mCi/mmol), or 200 μM [2-^{14}C]uracil (60 mCi/mmol). In some cases 50 mM succinate was also present. Termination, filtration, and counting were performed as described in Materials and Methods. The dotted line represents an estimation of the amount of [2-^{14}C]uridine that should be taken up based on the radioactive levels achieved when membranes are incubated with [U-^{14}C]uridine and succinate.*

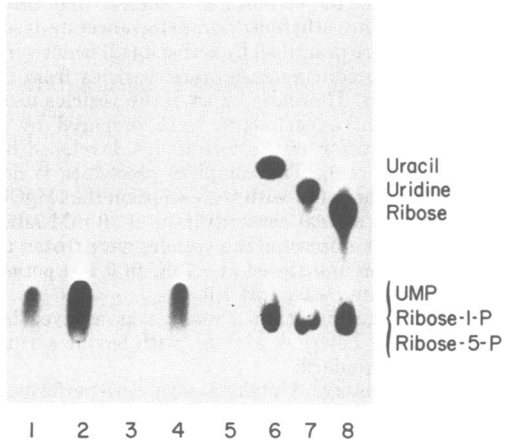

FIG. 2. *Autoradiography of a chromatogram of vesicle contents developed in solvent 2. Chromatography was performed on a cellulose thin-layer sheet. Lane 1, Vesicles incubated with uridine in the presence of succinate; lane 2, same as lane 1 plus ribose-1-P; lane 3, zero-time control for lanes 1 and 2; lane 4, vesicles incubated with uridine in the absence of succinate; lane 5, zero-time control for lane 4; lane 6, uracil and ribose-1-P; lane 7, uridine and ribose-5-P; lane 8, uridine 5'-monophosphate (UMP) and ribose.*

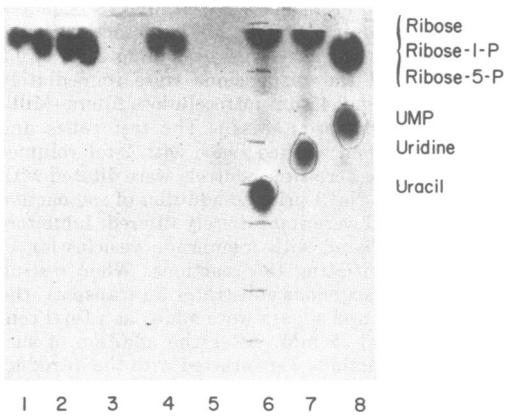

FIG. 3. *Autoradiography of a chromatogram of vesicle contents developed in solvent 9. See legend to Fig. 2 for type of thin-layer plate used and for identification of the spots in each lane.*

to co-migrate with ribose-1-P (Fig. 4). The trace amounts of ribose that are sometimes present are probably generated during the extraction of the vesicle contents.

A chromatogram developed in solvent 9 was subdivided into various segments for counting in a liquid scintillation spectrophotometer (Table 1). Only minimal radioactivity is associated with uracil, uridine, or uridine 5'-monophosphate. However, ribose-1-P is radioactively labeled and the radioactivity increases as a function of time. It may be concluded from these results that ribose-1-P is the major compound that appears within *S. typhimurium* membrane vesicles incubated with uridine.

Enhancement of ribose-1-P accumulation by energy sources. The presence of succinate markedly stimulates ribose-1-P accumulation (Table 2). This is a saturable process with a K_m of about 10 μM. A number of additional potential electron donors were screened to determine their relative efficiencies in energizing this ribose-1-P accumulation. The vesicles were derived from cells grown in the presence of glycerol. Whereas L-lactate, α-hydroxybutyrate, ascorbate plus phenazine methosulfate, D-lactate, and α-glycerophosphate stimulate uptake, maximal accumulation of ribose-1-P occurs in the presence of succinate.

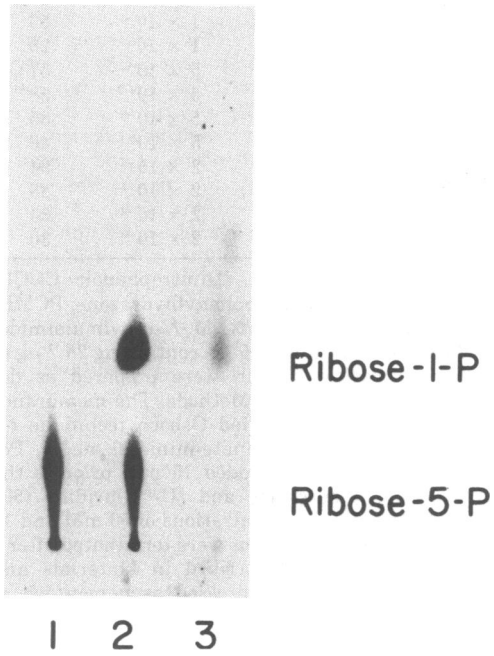

FIG. 4. *Autoradiography of a chromatogram of vesicle contents developed in solvent 27. A polyethyleneimine thin-layer plate was used in this experiment. Lane 1, Ribose-5-P; lane 2, ribose-5-P and ribose-1-P; lane 3, vesicles incubated with uridine in the presence of succinate.*

TABLE 1. *Chromatographic analysis of the contents of vesicles after incubation with [U-^{14}C]uridine and succinate*[a]

Relative position of standards on thin-layer chromatogram	Uptake of radioactivity (cpm)		
	0 min	3 min	10 min
1. Ribose-1-P --	67	1,033	3,155
2. ----------	42	62	245
3. UMP ----------	58	95	169
4. Uridine ------	127	112	154
5. Uracil --------	44	44	62
6. ----------	45	46	47
7. ----------	38	47	56
8. ----------	37	51	60
9. ----------	40	39	36

[a] The assay conditions were the same as indicated in the legend to Fig. 1, except that the reaction mixtures contained 41 μg of membrane vesicle protein, 50 mM succinate, and 10 μM [U-^{14}C]uridine (509 mCi/mmol). After extraction of the vesicle contents, the eluates were lyophilized and resuspended in 50 μl of distilled water. Portions of each sample (5 μl) were applied to a cellulose thin-layer sheet, which was chromatographed in solvent 9. Autoradiography and radioactive determinations were performed as outlined in Materials and Methods.

TABLE 2. *Effect of potential energy sources on ribose-1-P accumulation derived from [U-^{14}C]uridine*[a]

Energy source (20 mM)[b]	Amt of ribose-1-P accumulated (pmol per mg of protein/min)
None	54.0
Succinate	214.2
D-Lactate	108.1
DL-α-Glycerolphosphate	109.8
L-Lactate	89.3
DL-α-Hydroxybutyrate	82.3
Ascorbate + PMS	90.9

[a] Vesicles were prepared from cells grown in the presence of 0.1% glycerol. The reaction mixtures (100 μl) contained 83.6 μg of membrane vesicle protein and 10.6 μM [U-^{14}C]uridine (498 mCi/mmol). The potential energy sources were added 15 s prior to the addition of the radiolabeled uridine. Additional assay conditions are described in Materials and Methods.

[b] Except for phenazine methosulfate (PMS), which was 0.1 mM.

Other potential energy donors that were tested include phosphoenolpyruvate, malate, fumarate, citrate, DL-isocitrate, α-ketoglutarate, DL-β-hydroxybutyrate, glucose, adenosine triphosphate, flavin mononucleotide, flavin adenine dinucleotide, reduced nicotinamide ade-

nine dinucleotide, nicotinamide adenine dinucleotide phosphate, and 5-phosphoribosyl-1-pyrophosphate. All of these substrates were present at a final concentration of 20 mM with the exception of 5-phosphoribosyl-1-pyrophosphate, which was used at a concentration of 4 mM. The rate of ribose-1-P accumulation is not enhanced by any of these energy sources. Since succinate appears to be the most effective substrate for providing energy, it was included in most of the subsequent experiments.

Effect of inhibitors on succinate-stimulated ribose-1-P accumulation. Sulfhydryl reagents such as *p*-chloromercuribenzoate or *N*-ethylmaleimide inhibit succinate-stimulated ribose-1-P accumulation from uridine (Table 3). Inhibitors of electron transport or agents that can act as proton conductors like dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone also limit the amount of ribose-1-P accumulated by the vesicles. Thus, the succinate-stimulated accumulation of ribose-1-P derived from uridine appears to be coupled to electron transport.

Occurrence of intravesicular ribose-1-P derived from adenosine. Whereas *S. typhimurium* membrane vesicles take up radioactivity from [U-^{14}C]adenosine, they are incapable of incorporating radioactivity from [8-^{14}C]adenosine. The uptake, which appears to be limited to the accumulation of the ribose portion of the molecule, is stimulated by succinate. Upon chromatographic analysis, ribose-1-P is the only detectable intravesicular compound (Table 4). The results suggest that the ribose moiety of adenosine enters vesicles by a process similar to the entry of the ribose moiety of uridine.

Association of nucleoside phosphorylases with membrane vesicles. In the presence of inorganic phosphate, nucleoside phosphorylases convert nucleosides to ribose-1-P and free bases: nucleoside + $P_i \rightleftharpoons$ base + ribose-1-P. These phosphorolytic enzymes must be associated with *S. typhimurium* membrane vesicles, since incubation of vesicles with either uridine or adenosine in the presence of phosphate buffer results in the accumulation of ribose-1-P. To determine the specificity of such nucleoside phosphorylases, membranes were incubated with uniformly labeled nucleosides. After incubations for 10 and 20 min, the total reaction mixtures were applied to cellulose thin-layer sheets, and the various regions of the developed chromatograms were counted for radioactive content.

When vesicles are incubated with uridine, adenosine, or inosine, ribose-1-P and free bases are produced and their concentrations increase with time (Table 5). A small amount of inosine is detected in the adenosine reactions, apparently as a result of the presence of a membrane-associated adenosine deaminase. Thus, the vesicles have phosphorolytic activity for uridine, adenosine, and inosine.

Effect of exogenous sugars and bases on ribose-1-P accumulation. The accumulation of ribose-1-P within the vesicles could be due to the phosphorolysis of uridine in the external medium, with subsequent transport of ribose-1-P or one of its derivatives. If this were the case, the addition of exogenous ribose compounds should compete with the ribose-1-P derived from radiolabeled uridine. When added in a 125-fold excess over initial uridine concentra-

TABLE 3. *Effect of various inhibitors on succinate-stimulated ribose-1-P accumulation derived from [U-^{14}C]uridine*

Expt	Inhibitor[a]	Concn (M)	% Inhibition
1[b]	Rotenone	5×10^{-5}	50
		5×10^{-6}	23
	2,4-DNP	5×10^{-4}	66
		5×10^{-5}	45
	Antimycin A	1×10^{-4}	77
		1×10^{-5}	36
	CCCP	1×10^{-6}	59
		1×10^{-7}	45
	Oligomycin	1×10^{-4}	53
		1×10^{-5}	26
2[c]	KCN	5×10^{-3}	57
		5×10^{-4}	38
	Sodium azide	5×10^{-3}	85
		5×10^{-4}	46
	PCMB	2×10^{-4}	94
		2×10^{-5}	88
	NEM	2×10^{-3}	34
		2×10^{-4}	30

[a] Abbreviations: DNP, Dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide.

[b] Reaction mixtures (100 μl) containing 26.7 μg of membrane vesicle protein were prepared as described in Materials and Methods. The membranes were made by the modified Osborn technique (4) from cells grown in succinate-minimal media. Potential inhibitors were added 15 min prior to the introduction of succinate and [U-^{14}C]uridine (509 mCi/mmol) at final concentrations of 50 mM and 10 μM, respectively. Reactions were terminated after 4 min and assayed as described in Materials and Methods. Results are expressed as percentage of inhibition of ribose-1-P accumulation as determined from the uptake levels in the presence and absence of inhibitors.

[c] Assays were similar to those of experiment 1, except that 67 μg of membrane vesicle protein was present in the reaction mixtures. The membranes were derived from cells grown in nutrient broth and were prepared by the ethylenediaminetetraacetic acid-lysozyme procedure (12).

TABLE 4. *Chromatographic analysis of the contents of vesicles after incubation with [U-^{14}C]adenosine and succinate*[a]

Relative positions of standards on thin-layer chromatogram	Uptake of radioactivity (cpm)		
	0 min	3 min	10 min
1. Ribose-1-P -------	137	465	699
2. ----------	38	51	78
3. ----------	49	54	66
4. ----------	74	57	51
5. ----------	59	47	39
6. Inosine ----------	41	40	44
7. ----------	37	41	54
8. Adenosine:adenine ----------	101	160	85
	37	45	35
9. ----------			

[a] The reaction conditions were as in footnote *a*, Table 1, except for the presence of 9 μM [U-^{14}C]-adenosine (585 mCi/mmol).

tions, neither ribose, ribose-1-P, nor ribose-5-P significantly alters the kinetics of accumulation of uridine-derived ribose-1-P (Fig. 5). These results indicate that uridine is not metabolized outside the vesicles. Instead, cleavage by uridine phosphorylase must occur during transport or after intravesicular accumulation of the intact nucleoside.

Whereas the amount of uridine-derived ribose-1-P that appears within the vesicles is not affected by the presence of exogenous ribose compounds, there is a significant decline in the levels of ribose-1-P when various purine and pyrimidine bases are added to the incubation mixtures. The intravesicular ribose-1-P concentrations are reduced to the greatest extent in the presence of uracil and are least affected by the introduction of hypoxanthine.

Base-mediated efflux of preloaded ribose-1-P. The bases may exert their inhibitory effect either during the initial uptake process or after accumulation by causing efflux of intravesicular ribose-1-P. To differentiate between these possibilities, vesicles were preloaded with radiolabeled ribose-1-P by incubating them with uniformly labeled uridine. After the pre-accumulation of ribose-1-P, a purine or pyrimidine base was added to the reactions at a final concentration of 2.5 mM. This resulted in a simultaneous fivefold dilution of the original uridine substrate.

It can be seen in Fig. 6 that all of the added bases initiate an immediate efflux of intravesicular ribose-1-P. Maximal efflux is elicited in the presence of uracil, whereas hypoxanthine is the least effective base for inducing a loss of ribose-1-P. Thus, bases are capable of affecting the intravesicular levels of ribose-1-P derived from uridine by preventing the accumulation of this phosphorylated sugar. In contrast to the addition of exogenous bases, ribose-1-P or ribose do not have an influence on the amount of ribose-1-P retained by the vesicles. The slight depression in the ribose-1-P and ribose curves at 6 min was not observed in other experiments. The lack of exchange between extra- and intravesicular ribose-1-P indicates that the vesicles do not have a specific transport system for ribose-1-P and provides further evidence that uridine itself, rather than ribose-1-P, is the substrate that initially interacts with the transport system.

TABLE 5. *Presence of membrane-associated nucleoside phosphorylases*[a]

Substrate added	Radioactivity co-chromatographing with:	Radioactivity (cpm) in the reaction products after:	
		10 min	20 min
Uridine	Ribose-1-P	4,502[b]	7,833
	UMP	450	719
	Uridine	38,564	32,310
	Uracil	3,739	6,564
	----------	218	119
	----------	62	0
	----------	0	0
	----------	0	0
Adenosine	Ribose-1-P	5,726	9,692
	----------	136	247
	----------	0	4
	Inosine	229	985
	Hypoxanthine, AMP	153	205
	Adenosine, adenine	61,367	56,444
Inosine	Ribose-1-P	4,083	7,210
	----------	163	165
	----------	49	72
	----------	84	35
	Inosine	59,297	53,063
	Hypoxanthine	4,452	7,640
	----------	62	73
	----------	112	44

[a] Reaction mixtures (50 μl) contained 13.4 μg of membrane vesicle protein, 50 mM potassium phosphate (pH 7.0), and 10 mM magnesium sulfate. See Materials and Methods for the concentrations and specific activities of the nucleoside substrates. After termination of the reactions, 5 μl of each sample was applied to cellulose thin-layer sheets, which were developed in solvent 9. Autoradiographic and counting procedures were performed as described in Materials and Methods. UMP, Uridine 5'-monophosphate; AMP, adenosine 5'-monophosphate.

[b] All values have been normalized with respect to the total radioactivity in the zero-time controls.

Formation of nucleosides from uridine-derived ribose-1-P and exogenous bases. Since several nucleoside phosphorylase activities are associated with the vesicles, it seemed possible that exogenously added bases could combine with uridine-derived ribose-1-P to give rise to

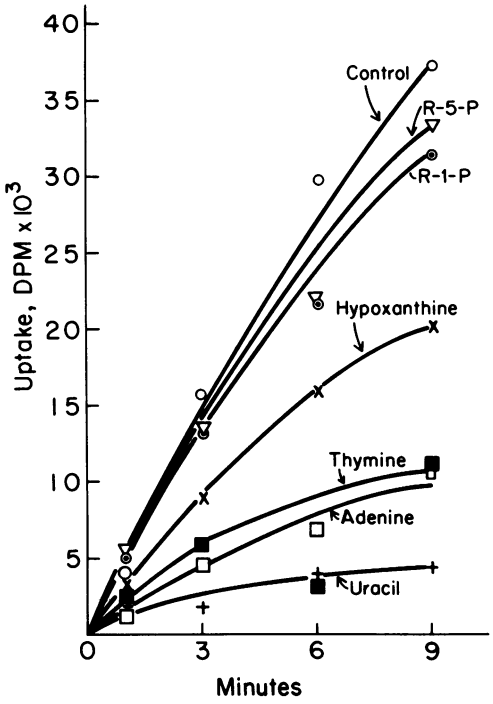

FIG. 5. *Effect of various sugars and bases on the accumulation of ribose-1-P by vesicles incubated with [U-^{14}C]uridine and succinate. The assay conditions were as indicated in the legend to Fig. 1, except that the reaction mixtures contained 20.5 μg of membrane vesicle protein, 50 mM succinate, and 10 μM [U-^{14}C]uridine (509 mCi/mmol). Although not shown, exogenous ribose had no effect on the amount of ribose-1-P accumulated. Symbols: ○, control; ▽, ribose-5-P; ⊙, ribose-1-P; ×, hypoxanthine; ■, thymine, □, adenine; +, uracil.*

new nucleosides. To determine if such nucleosides were being formed, total reaction mixtures containing uniformly labeled uridine plus a purine or pyrimidine base were subjected to thin-layer chromatography. The products of the reactions were located by autoradiography, and their radioactive levels were determined by liquid scintillation counting. The reactions were conducted with and without detergent. In the absence of either detergent or of any additional bases and sugars, uridine is converted to uracil and ribose-1-P (Table 6). This nucleoside has a total of nine carbon atoms, of which five represent the ribose portion of the molecule. Accordingly, about 5/9 or 56% of the total counts in the reaction products are associated with ribose-1-P.

The presence of exogenous adenine or hypoxanthine results in the appearance of new compounds identical by chromatography with adenosine and inosine, respectively (Table 6). These nucleosides, which apparently have only the ribose portions of their molecules labeled, are derived from radioactive ribose-1-P and the unlabeled bases. Hence, the total ribose-1-P produced in these reactions represents the sum of the radioactivity in both the ribose-1-P and the newly formed nucleoside regions. If the radioactivity associated with adenosine or inosine is considered, the amount of radioactivity associated with the total ribose-1-P produced in the presence of adenine or hypoxanthine, respectively, approaches the theoretical value of 56% of the total available radioactivity in the reaction products.

When uracil or thymine are added to vesicles

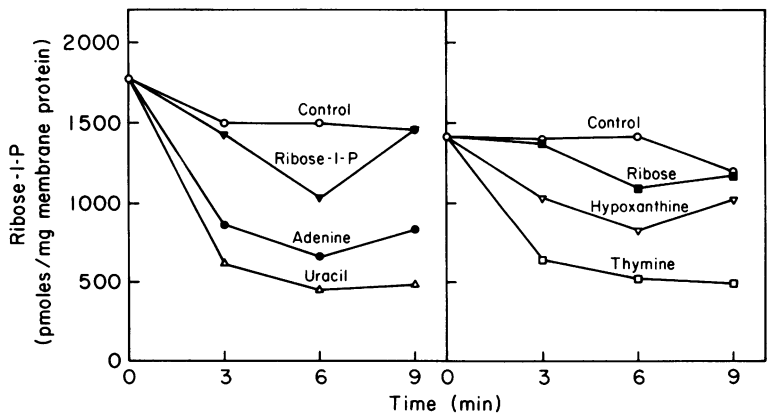

FIG. 6. *Base-mediated efflux of ribose-1-P from S. typhimurium membrane vesicles. The vesicles were preloaded with radiolabeled ribose-1-P and subsequently exposed to exogenous bases and sugars at final concentrations of 2.5 mM. The experimental procedures are detailed in Materials and Methods. Symbols: ○, control; ▼, ribose-1-P; ●, adenine; △, uracil; ■, ribose; ▽, hypoxanthine; □, thymine.*

TABLE 6. *Effect of various sugars and bases on the distribution of radioactive products found in the total reaction mixtures after incubation of vesicles with [U-^{14}C]uridine and succinate*[a]

Addition	Amt (cpm) of compound detected in the absence of Triton X-100			
	Ribose-1-P	Uracil	Inosine	Adenosine
None	3,758[b]	3,110	34	46
Adenine	2,088	3,891	26	2,478
Uracil	871	1,947	33	35
Hypoxanthine	2,641	3,055	738	152
Thymine	636	3,224	156	18
Ribose	3,140	2,956	75	67
Ribose-1-P	2,696	2,735	56	66
Ribose-5-P	3,321	2,996	0	161

[a] The reaction mixtures (50 μl) contained 10.2 μg of membrane vesicle protein, 10 μM [U-^{14}C]uridine (509 mCi/mmol), and other components as described in Materials and Methods. Reactions were terminated after incubation at 30°C for 10 min. A 5-μl portion of each assay was applied to cellulose thin-layer plates, which were developed in solvent 9. Autoradiographic and counting procedures were performed as described in Materials and Methods.

[b] Each assay was performed in duplicate and the results were averaged. In addition, all values have been normalized with respect to the total radioactivity in the zero-time control.

that are transporting uridine, the reaction mixtures contain less ribose-1-P than uracil. Some of the ribose-1-P derived from the splitting of uniformly labeled uridine is evidently converted to the corresponding nucleosides. Thus, the presence of exogenous uracil seems to lead to the formation of ribose-labeled uridine, but the amount synthesized cannot be quantitatively determined because of the large excess of unreacted uniformly labeled uridine. In addition, ribothymidine is synthesized from thymine and ribose-1-P. Since this nucleoside comigrates with uracil, both of these compounds contribute to the radioactivity located in the uracil region of the chromatogram.

The previous experiments involve an analysis of the total reaction mixtures. Separate analyses of the intra- and extravesicular compartments indicate that essentially all of the adenosine synthesized as a result of the presence of exogenous adenine is located outside the vesicles. The presence of Triton X-100 appears to inhibit the conversion of added bases to their corresponding nucleosides (Table 7). The inability to synthesize nucleosides when the membranes are solubilized with detergent could be due to inactivation of the enzyme(s) responsible for transferring a ribosyl group from uridine to the bases. However, when adenine is present at levels comparable to the previous experiments, along with a larger molar excess of ribose-1-P, adenosine reappears as a reaction product (Table 8). Thus, enzymatic activity is not abolished by the detergent. The results indicate that high levels of ribose-1-P are required for nucleoside synthesis. Since ribose-1-P is diluted by the entire reaction volume after membrane disruption, Triton X-100 may lower the effective concentration of this sugar, so nucleoside synthesis is not favored. These observations suggest that the formation of nucleosides by intact vesicles cannot take place in the external medium because the concentration of ribose-1-P would not be sufficient.

Effect of exogenous bases and sugars on uridine phosphorylase activity. The introduction of various bases into incubation mixtures containing intact vesicles may not only lead to the formation of new nucleosides, but may also have a direct inhibitory effect on the uridine phosphorylase reaction. With the exception of uracil, the effect of various bases on this enzy-

TABLE 7. *Effect of various sugars and bases on the distribution of radioactive products found in the total reaction mixtures after incubation of vesicles with [U-^{14}C]uridine and succinate in the presence of Triton X-100*[a]

Addition	Amt (cpm) of compound detected in the presence of Triton X-100[b]			
	Ribose-1-P	Uracil	Inosine	Adenosine
None	4,564	3,991	0	7
Adenine	3,562	3,113	21	16
Uracil	452	507	10	21
Hypoxanthine	2,870	2,696	38	0
Thymine	533	594	25	0
Ribose	3,859	3,356	0	0
Ribose-1-P	1,443	1,219	0	0
Ribose-5-P	3,350	2,914	0	0

[a] Reaction conditions were the same as those described for Table 6.

[b] Triton X-100 was present at a final concentration of 0.1%.

TABLE 8. *Effect of ribose-1-P levels on the ability of vesicles to synthesize adenosine in the presence of Triton X-100*[a]

Additions	Radioactivity (cpm) associated with adenosine
1. Triton X-100	707
2. Triton X-100 + 2.5 mM ribose-1-P	10,816

[a] Reaction conditions are the same as for Table 7, except adenine was radiolabeled (281 mCi/mmol) instead of uridine and 2.5 mM ribose-1-P was present in reaction 2.

matic activity can be assessed by comparing the total radioactivity in the reaction products of samples incubated in the presence and absence of added bases. The introduction of adenine causes an increase, whereas exogenous thymine leads to a decrease in the levels of radioactivity associated with the reaction products. Thus, the phosphorolysis of uridine appears to be stimulated by adenine and inhibited by thymine (Table 6). Although the amount of uridine that is reformed cannot be quantitatively determined, the presence of excess unlabeled uracil probably also exerts an inhibitory effect since the radioactivity associated with uracil is considerably below that of the control. This is not simply due to depletion of radiolabeled uracil during the resynthesis of uridine, because uracil and thymine, which are both substrates for uridine phosphorylase, would probably exhibit similar effects on this enzymatic activity. Thus, in the absence of detergent, uracil and thymine both appear to partially inhibit the uridine phosphorylase reaction. However, enough ribose-1-P is generated by the phosphorolysis of uridine so that some uridine and ribothymidine are synthesized when uracil and thymine, respectively, are added to the reactions. In contrast to the action of the bases, the presence of ribose, ribose-1-P, or ribose-5-P has only a slight effect on the phosphorolysis of uridine.

Upon dissolution of the membranes by Triton X-100, the uridine phosphorylase activity is enhanced (Table 7). The introduction of excess bases or sugars appears to inhibit the solubilized enzyme, with maximal inhibition occurring after the addition of uracil, thymine, or ribose-1-P. The magnitude of this inhibition is greater than that observed for reactions carried out in the absence of detergent and probably reflects the increased accessibility of the enzyme to the various effectors. Such an increase in sensitivity to inhibition by reaction products and other effectors has also been noted for related nucleic acid precursor metabolic enzymes in enteric bacteria (cf. 3, 6). Since nucleosides are not formed by the solubilized membrane preparations, the effect of the bases and sugars is to inhibit the phosphorolysis of uridine rather than to stimulate the synthesis of nucleosides by reversing the uridine phosphorylase reaction.

DISCUSSION

Ribose-1-P is essentially the only compound found within *S. typhimurium* LT2 membrane vesicles incubated with either uridine or adenosine. The appearance of this sugar arises from the phosphorolytic splitting of these nucleosides into ribose-1-P and corresponding bases (uracil and adenine) by membrane-associated nucleoside phosphorylases. Whereas ribose-1-P is accumulated, uracil and adenine are not found to any significant extent within the vesicles. They are either specifically excluded during the transport/phosphorolysis step or they rapidly equilibrate with the external medium.

In contrast to these observations, Hochstadt-Ozer (2) and Jackman and Hochstadt (10) have shown that bases derived from the extravesicular phosphorolysis of purine nucleosides are accumulated within *E. coli* and *S. typhimurium* vesicles as their nucleoside monophosphates (2, 6). This process is thought to be a group translocation mediated by membrane-associated phosphoribosyltransferases. In the present investigation, uridine uptake is not stimulated by 5-phosphoribosyl-1-pyrophosphate. This could be due to the fact that the bacterial cells from which the vesicles were derived were not grown in a medium (5) designed to induce very high levels of phosphoribosyltransferases. It is also possible that these enzymes are not retained by vesicles prepared by the procedures developed for this study (4).

Uridine is used as the transport substrate in most of the present experiments, because membranes incubated with adenosine accumulate lower levels of ribose-1-P and there is also evidence of adenosine deamination. The uptake kinetics of the ribose moiety of uridine is not significantly affected by the addition of exogenous sugars (ribose, ribose-1-P, and ribose-5-P). Therefore, the occurrence of intravesicular ribose-1-P is not dependent on the breakdown of uridine outside the vesicles with subsequent uptake of ribose-1-P or one of its metabolic derivatives. Instead, the intact nucleoside appears to serve as the transport substrate. The process leading from exogenous uridine to the accumulation of ribose-1-P shows saturation kinetics and is coupled to electron transport. Based on an intravesicular volume of 2 μl/mg of membrane protein for *S. typhimurium* vesicles (10), the levels of ribose-1-P that can accumulate within the vesicles as a result of the phosphorolytic cleavage of uridine may be as much as 100 times that of the initial uridine concentration.

Membrane-associated nucleoside phosphorylases are capable of catalyzing the synthesis of nucleosides from intravesicular ribose-1-P and exogenous bases. The formation of these nucleosides does not seem to occur in the external medium, because the ribose-1-P levels of solubilized membrane preparations are inadequate to

support nucleoside synthesis unless they are supplemented to approximate those of intact vesicles. The addition of bases also elicits an efflux of uridine-derived ribose-1-P. Thus, in the presence of exogenous bases intravesicular ribose-1-P is apparently converted into nucleosides, which then accumulate in the extravesicular compartment. This supposition is supported by the observation that essentially all of the adenosine that is formed by vesicles incubated with uridine and adenine is found outside the vesicles.

The synthesis of these nucleosides involves the passage of ribose from uridine to the added bases. In the case of ribonucleoside formation by vesicles incubated with uridine and pyrimidine bases (uracil and thymine), ribosyl transfer is probably accomplished by a reversal of the membrane-associated uridine phosphorylase reaction. Although not tested, thymidine phosphorylase may also be involved in the synthesis of ribothymidine. There are two possible explanations for the appearance of purine nucleosides. The ribose portion of uridine may be indirectly transferred to the purine bases by a reaction mechanism involving more than one phosphorylase (9, 19):

$$\text{uridine} + P_i \rightleftharpoons \text{uracil} + \text{ribose-1-P}$$

$$\text{ribose-1-P} + \text{purine base} \rightleftharpoons \text{purine nucleoside} + P_i$$

In the former case ribose-1-P serves as an obligatory intermediate. Alternatively, the ribose group may be directly transferred to the purine bases by a single phosphorylase (8, 9), as indicated in the formula: purine base + uridine $\rightleftharpoons$ uracil + Purine nucleoside.

It has been found that adenosine formation is inhibited when membranes are dissociated with detergent but synthesis of this nucleoside resumes after ribose-1-P levels are increased. Furthermore, vesicles preloaded with ribose-1-P appear to form nucleosides in the presence of exogenous bases. These observations suggest that ribose-1-P is an intermediate in the synthesis of nucleosides by the vesicles. Hence, ribosyl transfer from uridine to purine bases represents an indirect rather than a direct process involving the participation of coupled phosphorylases. An analogous reaction mechanism has been porposed for the passage of deoxyribosyl groups from thymine to adenine by *E. coli* cells (17).

The addition of uracil or thymine to intact or solubilized vesicles inhibits the phosphorolysis of uridine. In the absence of detergent, this reaction is not markedly affected by exogenous sugars (ribose-1-P, ribose-5-P, and ribose). However, ribose-1-P causes a marked inhibition of uridine cleavage when added to solubilized membranes. Thus, product inhibition of the membrane-associated uridine phosphorylase activity depends on which side of the membrane is accessible to the reaction products. The slight inhibition of this enzymatic activity by excess ribose-1-P in the absence of detergent is most likely due to a small population of incompletely sealed or inverted membranes.

Since the phosphorolysis of uridine can be inhibited or reversed from the inner side of the membrane by high concentrations of ribose-1-P, the uptake process for the ribose moiety of uridine may be self-limiting. This inhibition seems to be relieved by adding adenine to the reaction mixtures. Evidently, as intravesicular ribose-1-P is converted to extravesicular adenosine, more ribose-1-P accumulates within the vesicles as a result of additional uridine cleavage. The net effect is an apparent stimulation of uridine phosphorolysis in the presence of exogenous adenine.

Since both purines and pyrimidines are capable of limiting the levels of ribose-1-P that are accumulated, it is unlikely that all of these bases exert their inhibitory effect by competing with uridine for a common transport carrier. As shown in Results, the addition of exogenous bases to vesicles causes an efflux of pre-accumulated ribose-1-P in the form of nucleosides. In addition, some bases (uracil and thymine) strongly inhibit the uridine phosphorylase reaction. Thus, the ability of exogenous bases to interfere with the accumulation of ribose-1-P from uridine may be largely due to the ability to inhibit the uridine phosphorylase reaction and to bring about the synthesis of nucleosides, or both, which results in a lowering of intravesicular ribose-1-P levels.

Nucleosides may be actively transported and then phosphorolytically cleaved into free bases and ribose-1-P within the intravesicular space. However, this transport mechanism does not provide an adequate explanation for the exclusion of uracil and adenine from the vesicles. Furthermore, it is not certain how such bases, which are not accumulated by these *S. typhimurium* vesicles, can initiate the synthesis of nucleosides from intravesicular ribose-1-P which then appear in the extravesicular medium. To explain these observations, it would be necessary to postulate an additional mechanism for the rapid equilibration of the bases and newly formed nucleosides between the intra- and extravesicular compartments. However, these observations can also be explained by a group translocation mechanism in which the nucleoside phosphorylases span the membrane and have different ligand binding sites

on opposite sides of the membrane. Such an asymmetric arrangement for these enzymes is suggested by the ability of exogenous uracil or thymine to inhibit uridine cleavage, whereas ribose-1-P does not significantly affect this reaction unless the membranes have been disrupted. Thus, uridine phosphorylase, as well as other nucleoside phosphorylases, may assume a transmembranal orientation such that their binding sites for the base moieties are facing outward while the sites for ribose-1-P are directed toward the intravesicular compartment. Since the present evidence is consistent with such an asymmetric arrangement for the membrane-associated nucleoside phosphorylases, we suggest that the ribose moiety of nucleosides gains entry into vesicles by a group translocation process mediated by these enzymes. According to this translocation mechanism, nucleosides are cleaved during the membrane transport process. The resultant ribose-1-P is shunted to the intravesicular space while the bases are delivered to the external environment. The introduction of an exogenous base into reactions containing intact vesicles may lead to inhibition of the corresponding nucleoside phosphorylase activity or result in the accumulation of exogenous nucleosides derived from intravesicular ribose-1-P. Thus, this transport mechanism provides an explanation for the exclusion of bases during the uptake of the ribosyl moiety of nucleosides and for the ability of exogenous bases to induce an efflux of ribose-1-P coupled with the formation of new nucleosides. Based on this transport mechanism, nucleosides must be cleaved to ribose-1-P and free bases during transport in order to be taken up by *S. typhimurium* vesicles.

The postulated group translocation of the ribose portion of nucleosides has several unique features compared to other group translocation mechanisms. Instead of providing for a unidirectional accumulation of substrate (11, 13), the translocation of the ribose moiety can be reversed by the addition of exogenous bases. This is to be expected since the reactions catalyzed by the nucleoside phosphorylases are freely reversible. In addition, the uptake of ribose-1-P from nucleosides is coupled to electron transport. Perhaps, because of their reversibility, electron transport coupling is required to drive the reaction in the direction of uptake. Despite these new properties for a group translocation reaction, the evidence is consistent with such a transport mechanism. Thus, the nucleoside phosphorylases are responsible for the intravesicular accumulation of ribose-1-P from nucleosides. Similar observations on the specific retention of ribose-1-P and the exclusion of bases have been made for the uptake of several nucleosides by isolated mammalian plasma membrane vesicles incubated with several nucleosides (14, 20, 21). The present results also agree with a recent report (22) that ribose-1-P is selectively retained by intact *E. coli* B cells during uridine uptake.

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