THE ROLE OF THE MEMBRANE IN THE UTILIZATION OF NUCLEIC ACID PRECURSORS

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I. INTRODUCTION

Considerable detail on the routes, mechanisms, and regulation of purine and pyrimidine utilization in a diversity of organisms and cell lines, made possible in a number of instances by the availability of appropriate mutants has been elucidated since the subject was first reviewed in 1955[1] (and then only for *Escherichia coli*) and later further expanded in a textbook series in 1962. Because of the widespread usage of radioactively labeled exogenous nucleic acid precursors to trace the progress of cellular macromolecular synthesis it seems particularly important to evaluate the effects of at least some of the conversions involved in purine and pyrimidine utilization in terms of measurements that have been taken as indications of rates of nucleic acid synthesis. Moreover, these studies undertaken in mammalian systems relate intimately to the application of base analogs in chemotherapy.

This review will be limited to consideration of control mechanisms and interconversions prior to precursor entry into the nucleotide pool. An up-to-date account of the transport of bases and nucleosides and their conversion to nucleotides is presented; the further processing or utilization of nucleotides are, however, beyond the scope of this review.

II. BACTERIAL SYSTEMS

A. Purines

1. Purine Base Utilization

Conclusions concerning the mechanism of uptake of purine bases into bacteria – *Purine bases are thought to be taken up by a mechanism in which the appropriate phosphoribosyltransferase enzyme (a distinct one for each purine) attached to the membrane converts the purine to the nucleoside monophosphate in the very process of transporting it across the membrane. PRPP is cosubstrate for the reaction and may reach the enzymes' active site from either side of the membrane; a specific approach side has not yet been determined. The entire reaction is subject to feedback inhibition by numerous 5' nucleotides which seem to compete with PRPP. These enzymes are more loosely bound to membrane in that they can be solubilized by a variety of techniques and are at least catalytically active in*

aqueous solution. They are nevertheless considered true membrane components because of their intimate functional involvement in the purine transport process. The evidence upon which these conclusions are based is discussed in the account which follows.

a. Adenine Utilization

Uptake in *Bacillus subtilis* was investigated by Berlin and Stadtman[2] who found adenine uptake proportional to adenine phosphoribosyltransferase (PRT'ase) activity. Because the adenine phosphoribosyltransferase is inhibited by a number of 5′ nucleotide inhibitors, it was suggested that this might be the mechanism by which uptake is controlled. These authors also noted a stepwise pattern of periods of adenine uptake and plateaus over an 80-min time course which is suggestive of some "thermostat" type mechanism in the regulation, again implying the intermediacy of a regulatory process. Perhaps their most important finding was that the specific activity of intracellular AMP is greater than that of intracellular adenine after the cells are exposed to an environment containing ^{14}C-adenine. This by-pass of the internal adenine pool was taken as an indication of pool compartmentalization allowing external adenine to be converted to internal AMP without mixing with internal free adenine. Because the concentration of free adenine intracellularly was greater than that in the medium used, they concluded that "a transport system of high concentrative capacity for free purines is demonstrated." This conclusion is difficult to reconcile with the fact that the intracellular free adenine constituted 3 to 6% of the total radioactivity taken up and that this percentage increased with time rather than decreased, suggesting that it is a product rather than a precursor of intracellular metabolism. At the earliest measurement in these studies (50 sec), AMP represented about 29% of the nucleoside monophosphate, while at longer times it comprised as much as 48%. This result further suggests that much of the AMP may have arisen from interconversion of nucleoside monophosphates formed prior to AMP, but not involving free adenine. Such an interpretation is tenuous, however, because "other nucleoside monophosphates and some ADP" are not quantified in the presentation of data. Further difficulties in elucidating initial entry mechanisms in this study arise from the consideration that, by the time of the first measurement (50 sec), 90% of the total adenine taken up was already in the form of nucleoside triphosphates and nucleic acids.

The postulation of "a transport system of high concentrative capacity for free adenine"[2] is also difficult to reconcile with their observations that, of all the metabolic uncouplers and energy inhibitors tested, only the chelator 8-hydroxquinoline has dramatic inhibitory effects on adenine uptake. The authors did, however, attribute these results to an action of the quinoline on the phosphoribosyltransferase. Thus, concentration against a gradient was suggested without it being dependent on general metabolic energy.

These criticisms are in no way meant to detract from the importance of the contributions of this study at the time it was made. The recognition of the importance of the phosphoribosyltransferase in purine uptake and the finding in those that the phosphoribosyltransferases are regulated by a great many 5′ nucleotides (cf. Reference 2) which serve as a possible control mechanism for uptake are very significant achievements. These deductions laid the entire foundation for subsequent work which elucidated the way in which phosphoribosyltransferases might actually function in transport, compartmentalization, and regulation by so many effectors.

Adenine uptake in *E. coli* has been studied extensively by Hochstadt-Ozer and Stadtman.[3-5] The first aspect of their studies concerned with adenine phosphoribosyltransferase regulation of adenine uptake was an examination of regulatory controls operative on the activity of the enzyme itself. In order to establish findings unambiguously the adenine phosphoribosyltransferase was purified to homogeneity.[3] The starting material for the purification was homogenates of whole cells prepared from cultures completely dependent upon the purine phosphoribosyltransferases for meeting their purine requirements. They were grown in a medium that contained purine bases amethopterin (to block purine synthesis *de novo* as well as that of thymine synthesis), and replacement thymidine. Extracts isolated from cells grown in such a medium and harvested at the onset of the stationary phase contained as much as a hundred times the adenine phosphoribosyltransferase activity as extracts from control cultures grown on nutrient broth. Calculations made

with data obtained for the purified enzyme indicate the basal level of enzyme to be ~ 50 molecules per *E. coli* cell and at maximum derepression ~ 5,000 enzyme molecules per cell. The enzyme was purified from the extracts by conventional means and homogeneity was ascertained by constancy of specific activity over an effluent enzyme peak and by analytical acrylamide gel electrophoresis. The enzyme as freshly prepared at neutral pH and in the presence of Mg^{2+} has a molecular weight of ~ 40,000 daltons and is reasonably stable. Subunits with adenine phosphoribosyltransferase activity can be obtained at alkaline pH and in the absence of Mg^{2+}. Such smaller forms of the enzyme are, however, unstable, with almost all activity lost within 24 hr of dissociation, even at 4°C. The *E. coli* adenine phosphoribosyltransferase is specific for adenine, does not accept aminoimidazole carboxamide or 6-OH purines as substrates, and exhibits Michaelis-Menten kinetics. The 5′ nucleotides inhibit enzyme activity to varying extents, with all adenine 5′ nucleotides being potent inhibitors. However, adenosine, deoxyadenosine, 3′5′ cyclic AMP, and any free bases are ineffective. All nucleoside triphosphates, but only the purine nucleoside diphosphates are exceedingly effective inhibitors at submillimolar concentrations. Inhibition occurs via competition with 5-phosphoribosyl-1-pyrophosphate (PRPP). Though competition at the active site by all effectors sharing the 5′ phosphoribosyl moiety with the substrate PRPP would be a simple mechanism by which the entire interconvertible nucleotide pool might exert control on activity, there is some question as to whether there might not be several regulatory sites. Two reasons exist for raising this question. For one, uridine triphosphate (UTP) is a better inhibitor than ADP, for instance, whereas uridine diphosphate (UDP) does not significantly inhibit the reaction. Thus, both the phosphoribosyl configuration and base must be included in the structure necessary for control of enzyme activity. Secondly, certain combinations of 5′ nucleotides when present at physiological concentration are less inhibitory than each would be individually, suggesting a mechanism for antagonism (Hochstadt, unpublished results). Nucleoside triphosphates at very low concentrations stimulate activity but inhibit at higher concentrations.[3] Such stimulation may be related to induced conformational changes after effector interaction with either the catalytic or the regulatory site of one or more subunits of a complex enzyme, which affects the interaction of substrate and catalytic sites on other subunits in a positive manner (i.e., leading to increased activity).

Subsequent to the characterization of the homogeneous enzyme obtained from sonic extracts of *E. coli*, the possible role of enzyme in adenine phosphoribosyltransferase transport was investigated using isolated membrane vesicles prepared by the method of Kaback.[4,37] The membrane vesicles as isolated possess two enzymes capable of metabolizing adenine;† both adenine phosphoribosyltransferase and adenosine phosphorylase activities could be measured. However, when adenine uptake activity was assessed in the vesicles, only PRPP, the cosubstrate for the adenine phosphoribosyltransferase, had a significant stimulatory effect. Ribose-1-phosphate, the cosubstrate for adenine phosphorylase, was completely ineffective. When the vesicle contents were chromatographed it was observed that adenine, which appears to be concentrated relative to the medium, had accumulated intravesicularly as AMP and the AMP concentration within the vesicles reached many times the level of the original external adenine concentration. The kinetics of the uptake reaction in membrane vesicles closely parallel the kinetics of the membrane phosphoribosyltransferase, suggesting virtual identity of the membrane transferase with the enzyme purified to homogeneity from cell extracts.

Though inhibitors of the enzyme in vitro are also inhibitors of the transport reaction, on a qualitative basis, transport inhibition requires higher effector concentration to achieve similar inhibition. This finding suggests restricted access of the inhibitor to the regulatory site when the enzyme is embedded in the membrane. The fraction of enzyme which is purified with the vesicles can be eluted from the vesicles by freeze-thawing, with concomitant loss of uptake

†Both of these enzymes are more loosely bound than what is thought of as typical particulate insoluble enzymes usually called membrane enzymes. The major portions of the two enzymes can be obtained soluble from homogenates. Since they do purify with the membrane and moreover here show membrane function, they are considered soluble membrane constituents.

activity by the vesicles. The percentage of membrane-bound enzyme correlates well with uptake activity, although occasionally uptake activity could be diminished in excess of the amount of enzyme released. The phosphoribosyltransferase released cochromatographs with the completely purified enzyme isolated from cell homogenates and showed similar subunit and regulatory behavior. Two observations strongly suggest that free adenine must interact with the membrane enzyme before transport across the membrane occurs: PRPP is equally accessible to the active site when presented from within or without the vesicle[4] and the enzyme itself resides on the exterior of the membrane since it is shock-releasable.[5] It was therefore postulated that the membrane phosphoribosyltransferase is itself the transport system and that phosphoribosylation of adenine is a requirement for transport. Such requisite metabolism during transport is observed for a number of sugars in bacteria transported by the phosphoenolpyruvate-dependent phosphotransferase system[7] and these conversions have been termed group translocations. The group translocation mechanism could certainly explain all of the results of Berlin and Stadtman[2] relating to compartmentalization, by-pass of adenine pools, and ineffectiveness of general metabolic inhibitors on a specific concentrative uptake process. In particular, the latter observation concerning metabolic inhibitors may be explained by putative stores of PRPP in isolated membranes inferred by Hochstadt-Ozer[8] and by Hochstadt-Ozer and Stadtman.[4]

Several questions were unanswered, however, by the vesicle studies: a role for membrane adenosine phosphorylase, since it was not implicated in adenine utilization; the paucity of membrane-associated enzyme activity after membrane isolation in comparison with total cellular phosphoribosyltransferase; and the observation that more AMP is released on the exterior surface of the membrane vesicle after enzyme action than on the interior surface.

In order to answer these questions, the vesicle studies were followed up by investigations with intact cells.[5] Adenine uptake rates in whole cells reflect the participation of the total phosphoribosyltransferase in uptake under the reaction conditions expected to exist within whole cells. Thus, the membrane phosphoribosyltransferase (if it is assumed that *in situ* all of the adenine phosphoribosyltransferase is membrane associated) could be the only mechanism for adenine uptake by the cells. Moreover, regulatory effects of the metabolic pool could be quite easily demonstrated in intact cells. Freshly harvested cells could not be stimulated by glucose (or other energy sources) to take up more than at the basal rate observed in the absence of glucose nor could they be inhibited to take up less by nucleotide additions. Cells which had been starved in the cold for at least six hr, however, are responsive both to stimulation of uptake by a number of energy sources as well as to the inhibitory effect of 5′ nucleotides. Moreover, in the presence of an energy source and in the absence of nucleotide inhibitors, starved cells exhibit initial adenine uptake at a linear rate which exceeds the rate observed in freshly harvested cells. This higher than "freshly harvested rate" was attributed to release of the enzyme system from regulation as a consequence of depletion of the nucleotide pool during the period of starvation. In part, the response to effectors might also be attributable to greater access after some degree of starvation-related peptidoglycan autolysis.† Though several metabolizable sugars and sugar phosphates could serve as energy sources for the starved cells to resume uptake, only PRPP would stimulate adenine uptake in the presence of iodoacetamide. This was interpreted as indication that, with the exception of PRPP, all other energy donors require metabolism (presumably to form PRPP from ATP and ribose-5-P) involving the glycolytic pathway.

In starved cells of *E. coli* less than 10% of the adenine taken up in the first minute or two is recovered as nucleoside triphosphate or in macromolecules.[5] This is in contrast to the previously discussed studies of Berlin and Stadtman[2] in which 90% of the adenine taken up by freshly harvested cells of *B. subtilis* was found associated with such compounds even after 50 sec. Both in *E. coli*[5] and in *B. subtilis*[2] the predominant species of nucleoside monophosphates were not AMP. Though this was considered to result from either intracellular interconversions of the nucleotides or from reactions prior to uptake, there is considerable evidence now that the latter is clearly the

†Presumably the outer membrane of enteric bacteria does not present a selective barrier to the passage of small molecules from the environment whereas the inner membrane has specific and selection permeability (transport) properties.

case. Enzymes of base and nucleoside interconversion are now known to reside in the periplasm.[8] Moreover, interconversions in the periplasm are not subject to nucleotide pool regulation[6] while the intracellular enzymes for nucleoside monophosphate interconversion are subject to the most stringent pool regulation.[9] Furthermore, the addition of sodium sulfate, an inhibitor of the periplasmic purine nucleoside phosphorylase, to the reaction mixture during adenine uptake resulted in a significantly greater proportion of recovery of nucleoside monophosphate as AMP.[5]

These results may make it necessary to qualify the role of the membrane adenine phosphoribosyltransferase in adenine utilization in nature. Though there is sufficient enzyme to take up all the adenine by this mechanism, the majority of the adenine could possibly be taken up as hypoxanthine by a group of translocation to IMP in the absence of either guanine or hypoxanthine in the medium at the time of adenine uptake. The presumptive pathway would be adenine → adenosine → inosine → hypoxanthine → IMP. The enzymes for all the reactions are present in the membrane vesicle preparations and it is assumed that all but the last would take place on the outer surface of the membrane or in the periplasm while the last would proceed across the membrane itself. Of course, the experimental situation with adenine as solitary substrate may be artifactual; in nature if cells have any nucleic acid precursors to utilize, there may always be combinations of them, i.e., combinations of equal amounts of guanine and adenine as might be derived from nucleic acid substrates. In this case both the $6NH_2$ PRT'ase and 6-OH PRT'ase might be equally saturated and adenine might not be shunted through a pathway involving its deamination. In such instances independent feedback mechanisms for each might be fully operative. Nevertheless, if the intracellular pathway of adenine metabolism is to be exclusively followed, mutants for the periplasmic pathway between adenine and hypoxanthine[24] or for hypoxanthine uptake itself may be desirable as a means to further elucidating the pathway of direct adenine utilization.[10,11]

Though free bases are ineffective in regulating the activity of the purified enzyme, these do inhibit uptake in whole cells. It may be presumed that since the pathways for the uptake and phosphoribosylations of all free bases are functional, the free bases are only effective after conversion to nucleotides.

Because effectors are fully accessible from the outside even in whole cells under appropriate conditions, i.e., "shocking" them in by a transient osmotic shock opening and reannealing of vesicles gave no change in their effectiveness,[5] and only a small fraction of the adenine phosphoribosyltransferase is recovered on the membrane after membrane isolation, the possibility that the phosphoribosyltransferase is situated in the periplasmic space was considered. Numerous osomotic shock experiments were performed, the recovery of adenine phosphoribosyltransferase in the shock fluid was determined, and the extent of adenine uptake by shocked cells was measured. Each batch of cells gave a somewhat different proportion of enzyme release when shocked, but this was quite reproducible among duplicate shock treatments performed on portions of the same culture. The resulting loss in transport very closely parallels enzyme loss. This was the first reported instance of an enzyme which is neither quantitatively released into the shock fluid, as might be expected of a strictly periplasmic enzyme, nor completely retained as might be expected of an intracellular enzyme.[5] This behavior, however, is most consistent with the observations that the enzyme resides on the membrane itself, but located on the outer surface as indicated by effector and substrate access from the exterior. Thus, the association of the adenine phosphoribosyltransferase with the membrane is neither as tenuous as that of enzyme I and HPr of the PEP-dependent phosphotransferase transport system[7] for sugars, nor so tight that significant amounts (about half) cannot be released by osmotic shock (cf. Reference 5). Some of the major characteristics of the adenine phosphoribosyltransferase mediated adenine transport system in whole cells, vesicles, and purified enzyme are compared in Table 1.

B. Hypoxanthine and Guanine Utilization

Investigations similar to those for purification and classification of the adenine phosphoribosyltransferase of *E. coli* have been carried out with respect to the 6-OH purine phosphoribosyltransferase(s) (E.C. 2.4.2.7). These enzymes which transfer the 5-phosphoribosyl moiety for PRPP to guanine, hypoxanthine, and xanthine to form GMP, IMP, and XMP, respectively, have been purified[12-15] from a number of sources. The

TABLE 1

Characteristics of *E. coli* Adenine Phosphoribosyltransferase System and Adenine Uptake

	Adenine phosphoribosyltransferase		Adenine uptake	
	Purified enzyme	Membrane enzyme	Isolated membranes	Intact cells
K_m adenine	15-25 μM	15-25 μM	15-25 μM	15-25 μM
K_m PRPP	0.1-0.2 mM	0.1-0.2 mM	0.1-0.2 mM	0.1-0.2 mM
Nucleotide[a] effectors	ATP, dATP, ADP, GTP, GDP	ATP, dATP, ADP, GTP, GDP	ATP, dATP, ADP, GTP, GDP	ATP, dATP, ADP, GTP, GDP

[a]Several other nucleotide effectors were not tested in all four systems.

enzymes from enteric bacteria[12-14] and humans[15] have exhibited certain unusual properties. Though several enzymes (or isozymes) have been isolated, their relationship to one another remains unclear. The various forms of the human enzymes, separated by isoelectric focusing, differ in their activities for IMP, XMP, and GMP formation, though all three can be demonstrated as substrates for each form.[16] The relative activity for each substrate (hypoxanthine, xanthine, or guanine) of the isozymes changes as a function of storage at 4°C; the isoelectric mobility of the fraction correspondingly changes. Work in this laboratory[12] has resulted in the separation of several fractions of *E. coli* K_{12} 6-OH purine phosphoribosyltransferase(s) by chromatography on ECTEOLA-cellulose (in much the same way as was achieved by Krenitsky et al.[14]) We have found that *E. coli* enzyme fractions also change in substrate specificity when stored in the cold, but have not yet rechromatographed these fractions to determine whether alterations in elution patterns parallel activity changes (as was found for the human enzyme).[15,16]

In *E. coli*, the 6-OH purine phosphoribosyltransferase activities are derepressed when cells are made dependent on exogenous purines, as seen in Table 2. Derepression is coordinate for all purine activities and permits large quantities of the enzyme(s) to be obtained for purification and study. The derepressed activities are purified together throughout the procedures as shown in Table 3 and Figures 1 and 2. A subsequent purification step on ECTEOLA-cellulose (not shown) led to the isolation of four activity peaks, but the activity of these peaks is not always stable. Consequently, kinetic and regulatory data were obtained on the preparation prior to chromato-

TABLE 2

Derepression of 6-OH Purine Phosphoribosyltransferase Activities from *E. coli* K_{12}

			Specific activity of crude extract (μmol × 10^3/mg/min)		
Medium	Hours of growth[a]	Turbidity[b]	IMP formed	GMP formed	XMP formed
VBC Medium	14	130	0.041	0.046	0.054
	28	220	0.086	0.090	0.099
VBC Medium Plus Purines[c]	14	183	0.140	0.152	0.162
	28	277	0.611	0.654	0.701
PAT Medium[d]	14	147	1.649	1.710	2.078
	28	236	3.271	3.412	4.118

[a]Cultures were started from loop inocula and maintained with shaking at 37°.
[b]Klett units, #66 filter.
[c]Adenine, guanine, hypoxanthine, xanthine 0.1 mM each.
[d]Additions as for c. plus amethopterin, 0.3 μM; thymidine 0.1 mM.

Summary of Purification of [G, H, X] Purine PRTase Activity

Specific Activity for				
G → GMP	H → IMP	X → XMP	% Recovery	Comments
$\leq 5 \times 10^{-4}$	$\leq 5 \times 10^{-4}$	$\leq 5 \times 10^{-4}$		
$\sim 3.5 \times 10^{-3}$	3.3×10^{-3}	4×10^{-3}	<100	Interference by guanine deaminase, xanthine oxidase, and protease observed and possible cause of lack of precisely constant purification ratios
4.5×10^{-3}	3×10^{-3}	4×10^{-3}	100	
4.2×10^{-2}	4.8×10^{-2}	5.2×10^{-2}	70	
0.288	0.207	0.288	47	
1.17	1.09	1.46	40	Nine protein bands on acrylamide gels at pH 9.5
2.45	2.2	2.6	33	
8.0	7.0	8.0	23	Three to four protein bands on acrylamide gels at pH 9.5 All protein bands show [G,H,X] Purine PRT'ase activity.

TABLE 3 (Continued)

The procedure used for purifying the enzyme was as follows:

Step 1. Growth under conditions maximizing enzyme activity – See Reference 3 and Table 2.

Step 2. Preparation of cell extracts – Frozen cells were thawed at room temperature, suspended in 5 to 10 volumes of 50 m*M* potassium phosphate buffer (pH 7.5), and sonically disrupted at 0°C by six 30-sec bursts (at maximum output with Heat Systems-Ultrasonic, Inc., Model 185W Sonifier Cell Disruptor) with intervening cooling at 0°C and cell debris was removed by centrifugation at 10,000 rpm for 20 min.

Step 3. Streptomycin precipitation – Streptomycin sulfate at 4°C, 10% by volume of a 10% solution was added to the supernatant solution also at 4°C (20 to 40 mg of protein/ml) obtained from Step 2. After stirring for 10 min in the cold, the mixture was centrifuged.

Step 4. Ammonium sulfate precipitation – The supernatant solution from Step 3 was adjusted to a protein concentration of 10 mg/ml, pH 7.8, with tris-HCl (50 m*M* final) and a saturated solution of $(NH_4)_2SO_4$ was added to a final concentration of 35% saturation by volume. The treatment was at room temperature and equilibration was for 15 min with stirring. The precipitate was collected by centrifugation in the cold for 10 min at 10,000 rpm. The pellet was discarded. Saturated $(NH_4)_2SO_4$ solution was added to that supernatant solution to 42% saturation by volume. Equilibration and collection of the precipitate were as before; the precipitate was resuspended in 20 m*M* tris-HCl (pH 8.0). The fraction precipitating between 42% and 49% saturated ammonium sulfate was then collected and tested for adenine, guanine, hypoxanthine, and xanthine phosphoribosyltransferase activities; it was either pooled with the 35 to 42% fraction if it had considerable 6-OH purine transferase activity, used to prepare the adenine phosphoribosyltransferase if that activity predominated, or discarded. Figure 1 shows the distribution of the four purine phosphoribosyltransferase activities after $(NH_4)_2SO_4$ fractionation. It can be seen that the adenine phosphoribosyltransferase activity is clearly distinct from the other activities which coprecipitate by this means of fractionation.

Step 5. Precipitation with acetone – The protein fraction salting out between 35 and 42% saturated $(NH_4)_2SO_4$ (or 35 to 49%, see above) was adjusted to 10 mg/ml protein and 20 m*M* tris-HCl (pH 8.0). One volume of acetone at −7°C was added with stirring to four volumes of protein solution cooled in an ice-salt bath. The mixture was centrifuged and the supernatant brought to −10°C with an ice-salt bath; four additional volumes of acetone at −10°C were added with stirring and the mixture was centrifuged at −15°C. The pellet was collected, drained, and resuspended in 20 m*M* tris-HCl (pH 8.0) at 0°C (final volume – one half that of the protein solution originally treated with acetone), and dialyzed against one liter of 20 m*M* tris-HCl at 0°C for 2 hr.

Step 6. Treatment with Cγ gel – The solution from Step 5 was adjusted to pH 6.0 with 50 m*M* potassium phosphate buffer and mixed with a suspension of aged Cγ gel in 50 m*M* potassium phosphate buffer. Elution was at pH 7.5 and in some batches an additional pH 8.0 elution step was performed when recoveries at pH 7.5 were low.

Step 7. Chromatography on Biogel P150 – Step 7 was performed as described for adenine phosphoribosyltransferase in Reference 3. The 6-OH purine enzyme activities were recovered in the void volume.

Step 8. Chromatography on Biogel A 0.5 – Chromatography was performed under conditions employed for the other Biogel column in Step 7. These enzyme activities for all 6-OH purines cochromatographed on the A 0.5 column with the exception that a second lower molecular weight peak (activity unstable) was observed for the hypoxanthine to IMP reactions in some, but not all, preparations. Results are shown in Figure 2.

Assay of guanine phosphoribosyltransferase activity – Determination of activity was as described for adenine phosphoribosyltransferase.[3] Chromatographic separation of guanine and GMP was on cellulose thin layers in 1 *M* NH_4 acetate. Guanine and GMP have R_f values of 0.4 and 0.8, respectively, in this solvent.

Assay of xanthine phosphoribosyltransferase activity – Assay conditions were as described for the adenine[3] and guanine activities. Development of cellulose thin layers was in 1 *M* NH_4 acetate and the R_f values observed were 0.5 for xanthine and 0.85 for XMP.

Assay of hypoxanthine phosphoribosyltransferase activity – The convenient and rapid aqueous system described for separating the other purines from their nucleoside monophosphates had only limited use for separating hypoxanthine (R_f 0.6 in 1 *M* NH_4 acetate) from inosinic acid (R_f 0.9 in 1 *M* NH_4 acetate). It could be used with hypoxanthine of very high radiopurity if chromatograms were run for at least 10 cm and the reaction mixture did not contain other nucleoside additions. Therefore, for many of the kinetic and all of the regulatory experiments, the formation of inosinic acid was measured by separation from hypoxanthine by chromatography in a nonaqueous solvent system. The solvent used consisted of equal parts of the following two solutions, designated A and B (forms one phase at temperatures above 22°C). Solution A contained 1,246 ml of butanol and 84 ml of distilled water and solution B contained 620 ml of propionic acid and 790 ml of distilled water. Other details of the assay were as previously described for the adenine phosphoribosyltransferase.[3]

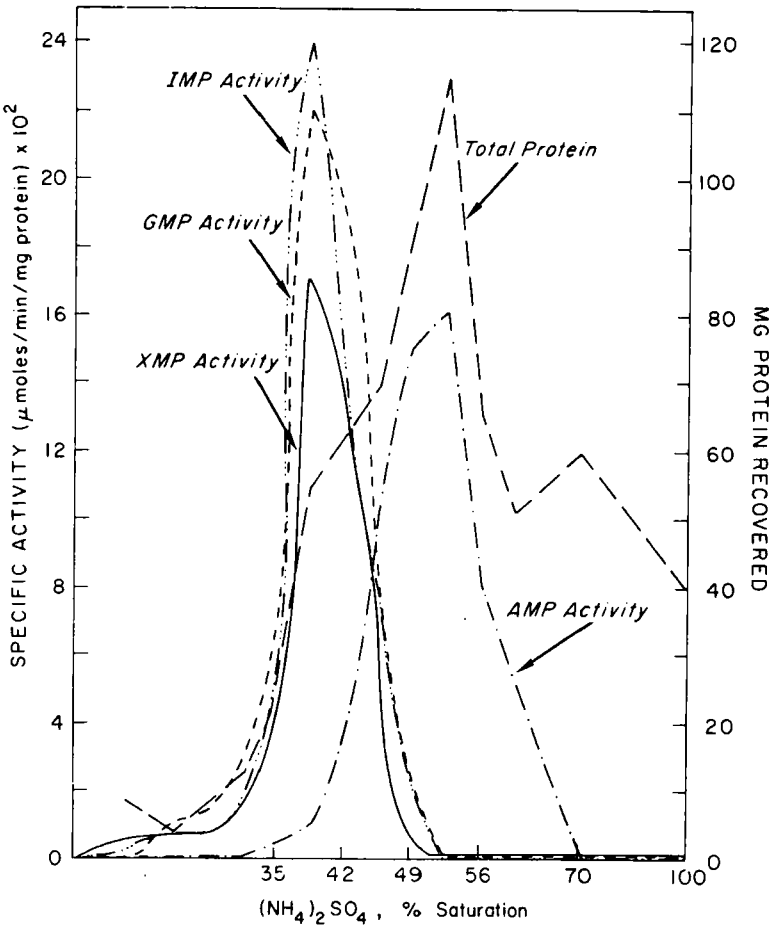

FIGURE 1. Separation of 6-OH and $6NH_2$ phosphoribosyltransferase activities in *E. coli.* (See Reference 3 and Table 3 for details.)

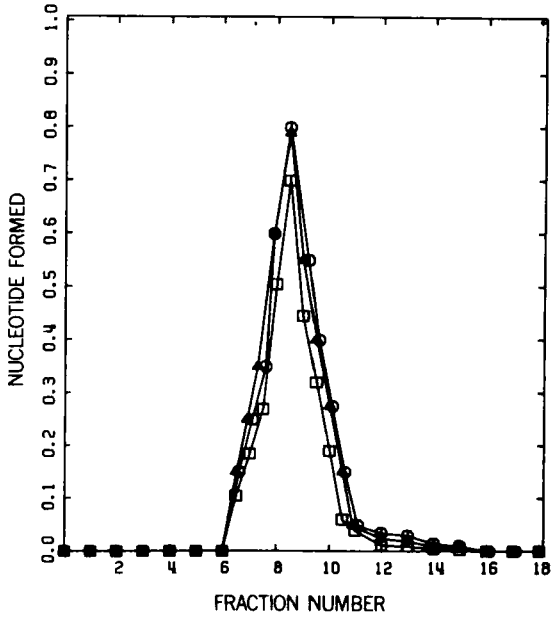

FIGURE 2. Cochromatography of xanthine, guanine, and hypoxanthine phosphoribosyltransferase activities in *E. coli.* (See Reference 3 and Table 3 for details.)

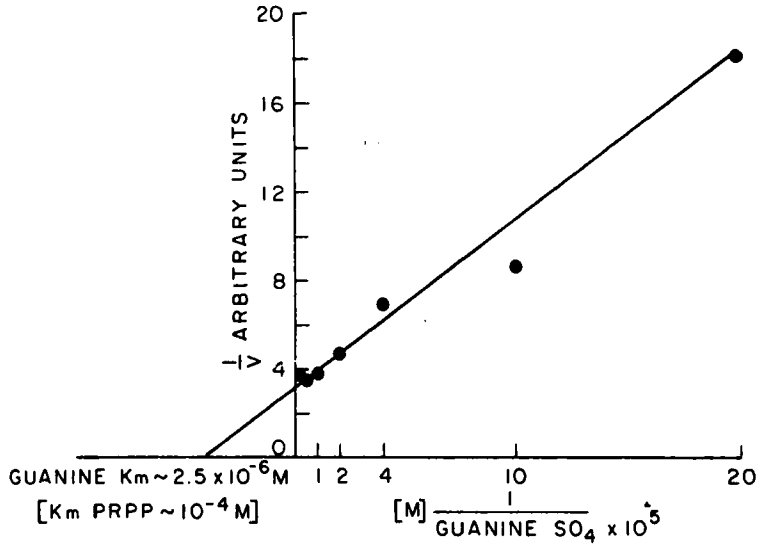

FIGURE 3. Rate dependence of guanine phophoribosyltransferase on guanine concentrations. Carried out as for adenine assay, Table 3 and Reference 3, the reaction was begun by the addition of PRPP, 2 m*M*µl final concentration. A parallel series containing the guanine concentrations indicated above, but to which no PRPP was added served as a control series which was subtracted from each experimental point. Enzyme activity of the fraction from Step 8, Table 3 is plotted as the double reciprocal of substrate concentration and reaction rate. Though the ordinate is plotted in arbitrary units specific activity at the y-intercept for each step in the purification is given in Table 3.

graphy on ECTEOLA-cellulose. In order to ascertain the number for isoenzymic forms of 6-OH purine phosphoribosyltransferase, such a preparation was subjected to gel electrophoresis; three to four activity bands were observed in several electrophorograms. Future experiments will examine the relationship of culture conditions on the number and mobility of isoenzymes found.

The enzyme kinetics for both PRPP and the purine substrates follow a Michaelis-Menten relationship (Figure 3). This typical kinetic response, together with the strict copurification observed, until the ECTEOLA step, leads us to believe that enzyme moieties probably share subunits or are otherwise related (cf. Reference 15). The pH profiles do differ for each substrate and give some indication of bimodality; maximal activity occurs at pH 7.9 for guanine and pH 8.8 for xanthine (Figure 4). Regulation of the enzymatic activities is quite pronounced in the presence of 5′ nucleotides. In the presence of saturating PRPP, however, unusual stimulation is observed with low concentration of 5′ nucleotides. Inhibition by 5′ nucleotides and stimulation at low effector concentration has been noted for the adenine phosphoribosyltransferase (previous section) and for other phosphoribosyltransferases.[3,8] Regulation of the XMP activity is seen in Figure 5, while control patterns for the GMP forming enzyme are summarized in

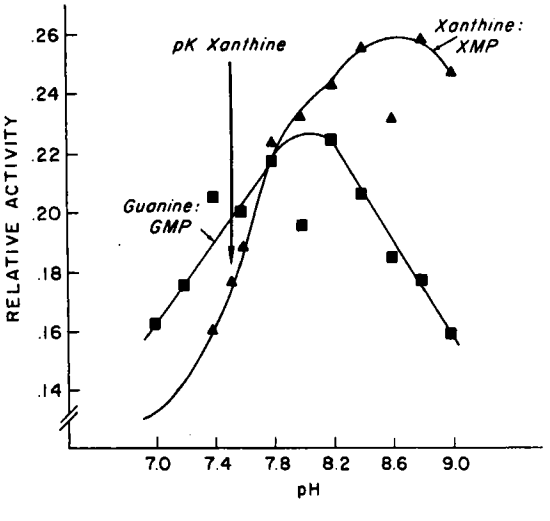

FIGURE 4. Effect of pH on 6-OH purine phosphoribosyltransferase. See Reference 3 and Table 3 legend for experimental details. The assay was performed using Tris-HCl 0.1 *M* final concentration.

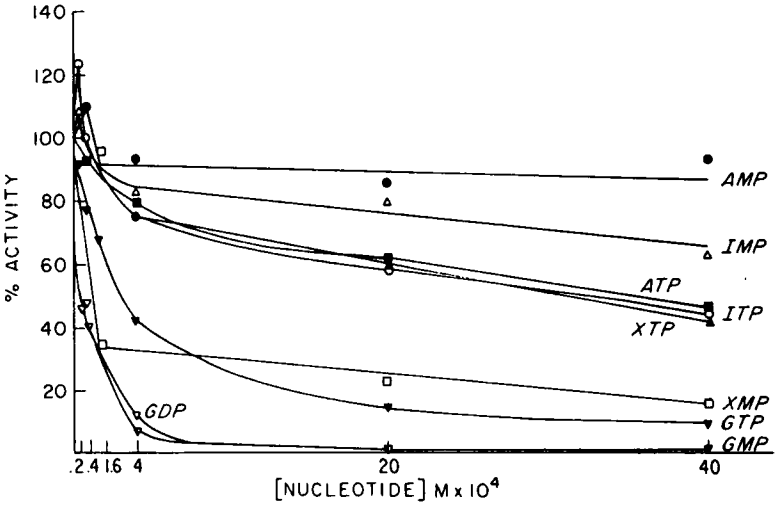

FIGURE 5. The effect of 5' nucleotides on xanthine phosphoribosyltransferase activity. Stock solutions (0.1 *M*) of the compounds tested were freshly prepared and neutralized to pH 6.0 to 6.5 with potassium hydroxide (where necessary) and stored in small aliquots at −20°C; any individual tube thawed twice was discarded after use. These reagents were first tested to migrate as a single spot in 1 *M* NH_4 acetate on cellulose thin layers. More dilute solutions were prepared as required, kept at 0 to 4°C and discarded after 24 hr. Reactions were performed as for the adenine enzyme described in Reference 3 with the exception that 0.2 m*M* 8-^{14}C-xanthine, 48 mCi/mmol (Schwarz Inc.) was the substrate. A measure of 20 ng of enzyme protein was used for obtaining each point and the pH for the reaction was maintained at 8.6 with Tris-HCl (0.1 *M* final).

Figures 6 and 7. Feedback regulation of both enzymes is similar to the pattern for the AMP forming enzyme[3] in that it is competitive with [PRPP] and that effectors with the same substituent in position 6 as the substrate are the most potent effectors. Some further information on the regulation of transport, which probably involves these enzymes, has also been obtained as a part of other studies on nucleoside uptake and 5-pyrophosphorylguanosine-3-pyrophosphate (ppGpp) regulation.[6,8]

It had been earlier observed that transferase activity for 6-OH purines was retained in mutants for which transport was defective, and this was at first taken as indication of a lack of involvement of PRT'ase in the transport process.[17] This view has recently been reevaluated in light of the finding that there are at least two forms of the enzyme.[10,11] Recently, two laboratories[10,11] have been able to isolate *Salmonella typhimurium* mutants either unable to utilize hypoxanthine while still able to utilize guanine[10] or conversely unable to take up guanine while retaining the ability to take up hypoxanthine.[11] One of the chromatographically identifiable enzyme forms is missing in this latter strain,[10] which contains a deletion in the pro AB region of the *Salmonella* chromosome and was found by accident rather than by selective techniques.[11] The selective techniques previously used[18] led to "adaptive resistance"† to 6-mercaptopurine rather than to a mutation, possibly because of the involvement of multiple gene products. However, the hypoxanthine-deficient strain was obtained by selection. It was constructed by an elaborate series of transductions beginning with double mutants for purine auxotrophy and then introducing defects in gua B and gua C cistrons which code for enzymes of guanine interconversion.[11] The map location of this new mutant, Hpt 1, has not yet been determined. Though each of these strains is completely unable to take up guanine or hypoxanthine, as the case may be, extracts of the cells do contain phosphoribosyltransferase activity for all

†Adaptive resistance is meant here as a change which depends on the continued presence of the selective agent for continued expression. The mechanism for the drug resistance in this instance has not been discovered.

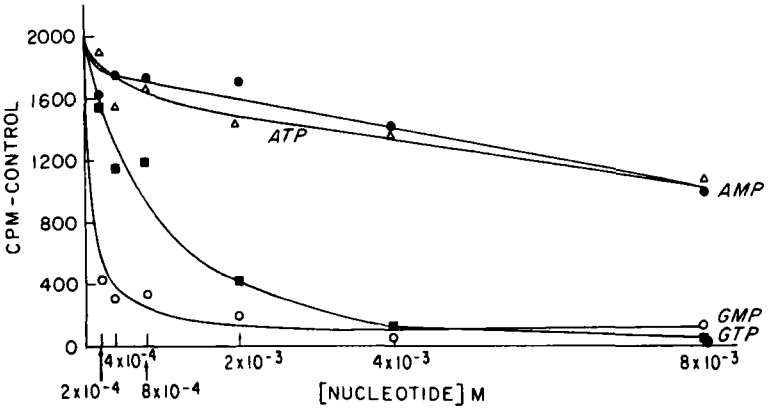

FIGURE 6. Comparison of the effects of guanine vs. adenine nucleotides on the activity of guanine phosphoribosyltransferase. The experiment was performed as described in Reference 3 and Figure 5 with the exception that 8-^{14}C-guanine 36.1 mCi/mmol (Amersham Searle) was the substrate tested. The pH was maintained at 8.4 in the presence of 0.1 *M* Tris-HCl. The reaction was allowed to proceed at 37°C for 5 min.

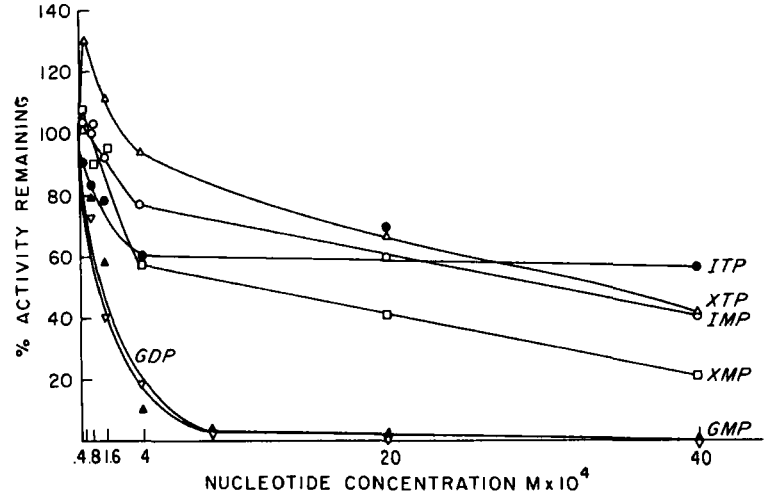

FIGURE 7. *Inhibition of guanine phosphoribosyltransferase by 5' nucleotides* of 6-OH purines. The experiment was conducted in the same way as in Figure 6 The total reaction mixture was 30 μl of which a 2 μl aliquot was spotted on the thin layer. Ten ng of enzyme protein was used in each experimental determination. Guanine in the amount of 0.4 nmol of the total of 5.6 nmol added was converted to GMP in the uninhibited samples in this experiment and in that described in Figure 6.

6-OH purines, albeit with lowered activity for the untransported substrate. A third variant has been recently described which is 6-mercaptopurine resistant, retains all guanine and almost all hypoxanthine activity, but no longer forms thioinosinic acid and lacks one form of the enzyme as detected by gel electrophoresis.[19]

Recently, evidence linking the enzyme forms on the membrane with transport has been obtained through the use of isolated membrane vesicles (Light and Hochstadt, unpublished results), prepared from the pro AB 47 deletion strain of Gots et al.[10] and the Hpt 1 strain of Chou and Martin.[11] Duplicate cultures were used for the preparation of sonic extracts and the purification of 6-OH purine phosphoribosyltransferase activity.

Isolated membrane vesicles could then be studied for both uptake and enzyme activity and compared with the enzyme prepared from whole cell extracts. Our results with either whole cell uptake or with the enzymes isolated from cell homogenates are identical to the results obtained in the laboratories originally describing the strains, i.e., there is virtually complete loss of uptake activity for one base, but only partial loss of catalytic activity by the soluble enzyme preparations. We then studied the vesicle preparations for both uptake and catalytic activity. The vesicles are very specific: those prepared from Pro AB 47 would only take up hypoxanthine while those from Hpt 1 would only take up guanine. When membrane phosphoribosyltransferase activity of the vesicles is measured independent of the transport assay, this specificity is retained: Pro AB 47 vesicles do not convert guanine to GMP, but do convert hypoxanthine to IMP; Hpt 1 does not convert hypoxanthine to IMP, but does convert guanine to GMP. When the vesicles were either subjected to sonic oscillation or to repeated freeze-thawing, significant enzyme activity could be liberated into a form that is unsedimented at a force of 100,000 times the force of gravity (one hr). These soluble enzymes are, however, capable of converting either guanine or hypoxanthine to the conjugate nucleotide. Thus, it appears that the same enzyme(s), when embedded in or associated with the membrane, have very restricted activity, but when released from the membrane have less restricted specificity. The conformation of these enzymes in the membrane, therefore, must be maintained by the environment.

It is also possible that the enzyme forms do not differ in their primary structure, but differ by having some lipid or glycan moiety attached to them which alters the single gene product 6-OH purine phosphoribosyltransferase. Alteration in a ligand could account for the change in the chromatographic behavior observed on ECTEOLA-cellulose columns and might also be the mechanism of enzyme binding to a specific membrane site. Thus, until sufficient characterization of the purified enzyme has been completed, it is possible that the mutant strains described[10,11] are altered in the ability to make or attach some specificity-rendering ligand rather than mutant in the structural gene(s) for the 6-OH phosphoribosyltransferase. This system seems to be potentially significant for study of membrane-enzyme interactions and elucidation of the possible lipid or glycan role in membrane protein function.

A possible regulation of hypoxanthine and guanine uptake concomitant to amino acid control of nucleic acid synthesis was suggested by the 90% contraction of the exogenously labeled guanine nucleotide pool during the stringent response.[8,11a] The specific relationship between uptake and pool contraction has recently been explored using isolated membrane vesicle transport systems and purified ppGpp.[8] During the stringent response (i.e., marked reduction of net macromolecular synthesis in stringent strains under conditions of starvation for one or more amino acids), stringent strains of *E. coli* do not utilize exogenous nucleic acid precursors for whatever RNA synthesis does proceed. To ascertain whether the regulatory mechanism is operative at the level of membrane transport, Hochstadt-Ozer and Cashel examined the effects of ppGpp, the unusual nucleotide synthesized during the stringent response, on isolated membrane vesicles and purified purine phosphoribosyltransferase activities. It was observed that membrane vesicle transport activity for 6-OH purines is inhibited over 80% in the presence of 3.0 m*M* ppGpp.[8] Intracellular levels of ppGpp during amino acid control are 2 to 4 m*M*; during exponential growth, ppGpp levels are 0.1 m*M* to 0.4 m*M*. The effects of ppGpp on the membrane-bound phosphoribosyltransferase activity for IMP and GMP formation are almost identical to the effect on transport. The effect of ppGpp was also tested for its effects on the activity of purified soluble enzyme in forming GMP and IMP from guanine and hypoxanthine respectively. The effects on the soluble enzyme are far more dramatic than those observed for ppGpp interaction with the membrane. Eighty to ninety percent inhibition of GMP formation by purified enzyme was observed at ppGpp concentrations usually found within the cell during exponential growth. Since cells can be made dependent on exogenous guanine, for instance, and thus would be required to utilize the phosphoribosyltransferase at almost full capacity (cf. calculations in Reference 5), the fact that the inhibitions observed in vitro are inconsistent with similar functions of a cytoplasmic soluble enzyme in vivo provides additional, albeit indirect, evidence that all purine phosphoribosyltransferase activity *in situ* must be membrane-associated. The membrane association in this case undoubtedly either maintains the

enzyme in a conformation that renders it less susceptible to effector interaction, or physically precludes access by ppGpp. From the complex kinetics of ppGpp inhibition, the former possibility may be the more likely.[8] A similar relationship of soluble and membrane-bound activities was observed for the effects of ppGpp on the adenine enzyme and uptake system. Though ppGpp has, overall, far less effect on 6-NH_2 purine utilization than on 6-OH purine utilization, the inhibiting effects on the isolated adenine phosphoribosyltransferase are at least twofold greater than those observed with the membrane-bound enzyme. The lesser effect on 6-NH_2 purine utilization is also parallel in vivo during the amino acid control phenomenon,[11a] by a lesser effect on ATP pool labeling but exogenous precursors than on the GTP pool labeling.

Though xanthylic acid formation has been observed in 6-OH purine phosphoribosyltransferase preparations,[12,15] and can serve as a source of required purine in certain strains, no xanthine uptake has been demonstrated by isolated vesicles despite repeated attempts to observe such transport.[4,12] Gots et al.[10] found that their deletion strain Pro AB 47 lacks both guanine and xanthine uptake and phosphoribosyltransferase activity. Since the Pro AB 47 strain is a deletion, the possibility exists that the deletion encompasses related but distinct functions for xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase which may be closely linked (and may even have arisen from gene duplication). This explanation would reconcile the conflicting results in *E. coli*[4,12] in which guanine uptake, but not xanthine uptake, can be observed with vesicles. A distinct xanthine uptake and enzyme system may exist which could be far more susceptible to loss by osmotic shock or during vesicle preparation than the guanine system. The complete loss of uracil[20,21] and orotate (Hochstadt, unpublished results) phosphoribosyltransferase(s) during vesicle preparation has been noted. In *Pseudomonas aeruginosa* a separate mechanism for xanthine and hypoxanthine uptake has been described; however, no determination of guanine uptake was made in that study.[22]

2. Purine Nucleoside Utilization

Conclusions concerning purine nucleoside uptake into bacteria – *The predominant mechanism of purine nucleoside uptake involves two enzymatic steps. The first is the cleavage of the nucleoside to a free base and ribose-1-P. This reaction is thought to occur either on the membrane or in the periplasm. The nucleoside phophorylase like the phosphoribosyltransferases can be easily solubilized and released from the membrane but is also found to be attached to the membrane in other instances. This reaction is not subject to feedback control. The ribose-P generated in the reaction may be released directly upon cleavage to the inner surface of the membrane. The free base is released to the periplasm and is then taken up in a second reaction mediated by the appropriate phosphoribosyltransferase and PRPP as any free purine base would be. This second reaction results in intracellular nucleoside monophosphate which occurs across the membrane and is feedback controlled by 5′ nucleotides. A secondary mechanism, one in which the nucleoside is taken up intact appears possible when this primary pathway is not functioning since a few mutants lacking nucleoside phosphorylases seem able to use purine nucleosides. The predominant class of mutants lacking the purine nucleoside phosphorylase to cleave the nucleoside as reaction 1 are unable to use purine nucleosides at all, however. The experimental data upon which these conclusions are based are as follows:*

a. Adenosine Utilization

Hochstadt-Ozer and Stadtman had observed that adenosine phosphorylase is present on isolated membrane vesicles, but does not participate in the free utilization of adenine utilization.[4] The enzyme had previously been localized in the periplasm, since it was found by Munch-Petersen to be releasable by shock.[23] Several other studies further suggested that the phosphorylase might be necessary for adenosine utilization; mutants unable to use purine nucleosides for meeting auxotrophic purine requirements, but still capable of growth on the purine-free base were repeatedly characterized as purine nucleoside phosphorylase deficient.[24,25] Moreover, the earlier classic work of Roberts et al.[1] had sugggested that nucleoside scission might be a requirement for utilization. The Carnegie group found disparate specific radioactivity recovered in the adenine and ribose moieties of nucleic acid adenylate residues when similar specific radioactivity would have been expected had the nucleoside been utilized intact. It was therefore concluded by these workers that purine

nucleosides are not used as units for nucleic acid synthesis, but rather are cleaved to yield a readily utilized purine fragment.[1] Peterson et al. observed that adenosine was first rapidly deaminated to inosine and this in turn cleaved to yield hypoxanthine in *E. coil.*[26,27] The hypoxanthine thus formed was thought to then be utilized more slowly for nucleotide formation. Such utilization of adenosine is supported by the studies of Zimmerman and Magasanik[28] who found that an adenine requiring mutant of *Salmonella typhimurium* deaminated adenosine very rapidly and grew very slowly in medium containing adenosine as a substitute for adenine. Studies reporting adenosine uptake into whole cells must therefore be viewed in terms of whether or not a specific adenosine transport mechanism per se is being studied. The characteristics of an inosine or hypoxanthine transport mechanism could in fact be what is examined, described, and simply termed "adenosine transport."

There are several major factors which undoubtedly determine whether adenosine will be deaminated as the first step in its utilization: the degree to which a given bacterial strain possesses a functional adenosine deaminase, the level of derepression of that enzyme along with several other periplasmic enzymes for the scission and separate processing of the base and sugar moieties,[23,29] the relative proportions of bases and/or nucleosides in the medium competing for the several alternate pathways, the intracellular nucleotide pool composition which could favor one alternative pathway over another, the activity levels (considering repression and retroinhibition) of nucleotide interconverting enzymes (e.g., GMP reductase), and the status of histidine biosynthesis. It will be remembered that histidine synthesis requires the replacement of carbon 2 of AMP by a single carbon fragment and comprises one more route from exogenous adenine or adenosine to cellular IMP and GMP.[30,31] There is however a mutant blocked between AICAR (Amino imidazolecarboxamideribotide) and IMP which can synthesize histidine so that this pathway seems not to be the only possible route of histidine biosynthesis (B. Magasanik, personal communication).

Thus, though there is no question that a significant fraction of adenosine in the medium is converted to inosine prior to transport, any attempts to study adenosine transport specifically, must be conducted in the absence of deaminase, as in *E. coli*[24] or *Salmonella* mutants,[25] or with osmotically shocked cells or isolated vesicles which have substantially lost the deaminase activity.

Adenosine uptake has been studied in isolated vesicles of *E. coli* by Hochstadt-Ozer.[6] Experiments in which the uptake products were characterized used 8-^{14}C adenosine exclusively, because the ribose moiety from nucleosides seems to be utilized by independent mechanisms in a number of instances, as will be discussed in the section on pyrimidine nucleoside utilization. When uptake activities of 8-^{14}C adenine and 8-^{14}C adenosine by isolated vesicles are compared, they seem identical in numerous ways. Both are stimulated by PRPP and exhibited the same concentration dependence. Extravesicularly, adenosine is rapidly converted to adenine (59% found as free adenine, less than 0.5% as inosine, and none as hypoxanthine after one minute incubation).[6]

The rate of substrate leakage from the vesicles at 40°C after uptake at 30°C is identical whether adenosine or adenine is offered in the uptake assay. Similarly, the temperature dependence of accumulation of radioactivity between 0°C and 50°C is identical for 8-^{14}C adenosine or 8-^{14}C adenine. Moreover, retroinhibition by AMP, ATP, and GTP are also identical. ATP, furthermore, does not inhibit the interconversion of adenine and adenosine via the purine nucleoside phosphorylase. In the conversion of adenosine to AMP via adenine, the adenine PRT'ase reaction was shown to be the rate limiting reaction of the two enzymatic steps (PRT'ase and nucleoside phosphorylase). Thus, the identical regulation for conversion of external adenosine and adenine to internal AMP supports the notion that, in the absence of deamination, adenine is a requisite intermediate in AMP formation from adenosine. Finally, repeated attempts to identify adenosine kinase which could mediate adenosine conversion to AMP without an adenine intermediate formation of adenine in cells and membranes from enteric bacteria have been completely unsuccessful.[6,25]

Thus, all adenosine not deaminated in vivo is proposed to be utilized by a two-step reaction, initially involving conversion to adenine by the periplasmic purine nucleoside phosphorylase, and then group translocation across the membrane by the adenine phosphoribosyltransferase. Such a model is in conflict with other suggested mechanisms for adenosine uptake based upon results

with whole cells[26,27] but these studies may partially represent utilization of inosine or hypoxanthine resulting from deamination. Indeed, in these studies the rapid conversion is noted, but the periplasmic compartment was not yet appreciated and metabolism was presumed to be subsequent to uptake. In a more recent study[32] where the role of the periplasmic enzymes was appreciated, transport of deoxyguanosine, deoxycytidine, and showdomycin is compared in shocked and unshocked *E. coli*. Though the paper attempts to conclude that two systems for uptake of adenosine as adenosine exist, there are serious flaws in the completeness of the data and logic to substantiate the conclusions. The only uptake experiments reported for adenosine utilization in this study involved nucleoside competition experiments, kinetics, and the effect of metabolic inhibitors on uptake in the wild-type strain and in a showdomycin resistant mutant.[32] After six min exposure of cells to adenosine, substantial conversion of extracellular adenosine was noted: only 15% of the label remaining outside (~85% of the total) was still adenosine in the sensitive strain and 45% was still adenosine in the mutant. Large amounts (greater than half in the wild-type) appear as inosine in the filtrate as well as in the form of another unidentified product (likely hypoxanthine). This result is consistent with all other reports[24-29] and may even underestimate the great extent to which such metabolism occurs in the periplasm; periplasmic metabolism might in a way be considered as metabolism occurring outside the cell yet it would not be apparent in assays of the culture filtrate, the usual test of reactions occurring outside the cell or at least outside the cell membrane. Though these results appear at the beginning of the paper, the authors seem to ignore their own findings and postulate not one, but two systems for adenosine uptake without characterizing true substrate(s) and product(s). With the observations that uptake of radioactivity from 8-^{14}C adenosine shows bimodal saturability when plotted in double reciprocal fashion, and that uptake in the resistant mutant is subject to inhibitory patterns different from the parent, the authors postulate the existence of two adenosine transport systems. Although data were presented indicating that after a few minutes the substrates available for uptake are very different for parent and mutant, which could explain the different uptake in the two strains, this seems to have been overlooked. The results of each experiment in this paper can be most simply explained by the action of showdomycin as an inhibitor of deamination prior to uptake. Cytidine, which also undergoes extensive deamination, was similarly studied, and the same conclusion of multiple systems was reached.

Thus, the evidence for adenosine transport (intact) into whole cells must await further study with deaminase-minus mutants and must include osmotic shock experiments.†

One paradox does arise, however, from the otherwise rather clear-cut studies with adenosine utilization in vesicles.[6] If, as it appears, the adenine phosphoribosyltransferase were the rate-limiting step in adenosine utilization, then K_m for the overall reaction from external adenosine to internal AMP should be no lower than that of the rate-limiting reaction. In fact, the apparent K_m would be expected to be greater since the accumulation of free adenine would be expected to influence the rate of the second and apparently rate-limiting step. The experimental results, however, indicate just the opposite; the overall system is saturated at lower concentrations of adenosine than of free adenine. This observation may indicate that purine nucleoside phosphorylase-complexed adenine rather than free adenine is the preferred substrate for the adenine phosphoribosyltransferase reaction or possibly a complex continuum of conformational changes of the enzyme is induced when others in the same pathway are catalytically active. Either of these hypotheses is most easily envisioned when enzymes in a pathway are physically associated with one another in a functional complex as might exist on the membrane. That this may in fact be the case is suggested by the many observations of functional differences of these nucleoside-

†B. Jochimsen has just informed us from Copenhagen that a new class of mutants in *E. coli* K_{12} has been isolated which can use purine nucleosides without purine nucleoside phosphorylase. One of these mutants, Sφ405, also lacks adenosine deaminase and thus is postulated to take up adenosine intact. Thus, Jochimsen seems to have uncovered a second adenosine utilization mechanism that may only operate when the predominant mechanism seen in previous mutants[24,25] and also seen on isolated vesicles,[6] is lacking.

metabolizing enzymes in vivo and in extracts.[6,23,24,33]

b. Inosine and Guanosine Uptake

Inosine uptake was studied by Peterson, Boniface, and Koch[26] who assumed that, similar to adenosine "uptake" and excretion as inosine, inosine was "taken up," excreted as hypoxanthine, and that this hypoxanthine was then "taken up." That this series of events with each representing a transport process was suggested to occur, was quite logical at the time. Neu and Heppel[34] had reported a shock releasable fraction of cellular constituents only months before, and the concept of the periplasm had not yet been defined. Consequently, metabolism of substrates by cell-associated (as opposed to exo- or excreted enzymes) was assumed to take place subsequent to transport. The findings by Peterson and Koch of intermediates excreted into the medium and then reutilized, rather than remaining cell-associated in the periplasm prior to transport, is undoubtedly an artifact of the ice-cold washes that were repeatedly used in an attempt to instantaneously stop the reaction. Cold shock seems quite effective in releasing small molecules into the medium[35] and may also account for the adenosine conversion observed in the filtrates in the work of Komatsu and Tanaka[32] mentioned earlier, as these workers also stressed that several ice-cold washes were employed in their procedure. In more recent studies with whole cells, warm (37°C) or ambient temperature washes have been employed to overcome the problem of cold-shock leakage.[5,10,11,63a]

The data presented by Peterson and Koch,[27] though explained as utilization of inosine via conversion to hypoxanthine, also suggest that considerable free inosine can accumulate in the cells under certain conditions, i.e., in the presence of caffeine. The work of Hoffmeyer and Neuhard[25] supports the idea that inosine might be taken up intact in addition to its predominant mechanism of uptake via hypoxanthine. Their *S. typhimurium* mutant, JH 48, a purine auxotroph which also lacks purine nucleoside phosphorylase, can grow with inosine as the sole purine source, suggesting the presence of an inosine kinase. The extent to which this pathway can function is limited, as a low growth rate was observed, even of adenine and histidine.[25] This suggests inosine or adenine transport inhibition caused by utilization of the putative inosine kinase pathway, rather than by a low activity of the enzyme, as being responsible for slow growth under the conditions employed.

A comparative study of hypoxanthine and inosine uptake was undertaken by Hochstadt-Ozer[6] using isolated vesicles prepared from *E. coli* K_{12}, to firmly establish the metabolic conversions implied by the earlier studies.[25-28] It was found that inosine is converted to hypoxanthine by a membrane-localized or periplasm-localized† purine nucleoside phosphorylase. Like adenosine phosphorylase, the reaction shows sigmoid kinetics with respect to cleavage of the ribosidic bond, with liberation of the free base disfavored at low nucleoside concentration. But unlike the situation with the adenosine phosphorylase, the very rapid conversion to free base is not as strongly favored kinetically. Temperature dependent uptake and efflux are identical for hypoxanthine-8-^{14}C and inosine-8-^{14}C. Inhibition of uptake and of IMP formation by GMP, GTP, or ATP are also identical when either inosine or hypoxanthine is the substrate. The lower K_m for the overall conversion of inosine to IMP, i.e., lower than that for the second step: hypoxanthine → IMP, suggests that the reactions are catalyzed by an enzyme complex incorporating positive cooperativity, was suggested previously for adenosine to AMP conversion proceeding via the free base (cf. Reference 6 for discussion of positive cooperativity in this system).

Deoxyguanosine uptake in *E. coli* was also studied by Komatsu and Tanaka.[32] In the wild-

†When membrane vesicles are isolated and are found to possess particular enzyme activities, one of three origins of such enzymes is possible. The enzymes can be integral parts of the membrane itself, they may be more loosely associated as in the case of the so-called "shock-releasable" or "periplasmic" enzymes or they may be simply adsorbed during the preparative procedure of isolating the vesicles. The enzymes, however, are assumed to fall into the first two categories if they are found to be shock-releasable from whole cells and when they purify with the membrane and participate in a biochemically known pathway of reaction observed to occur *in situ* in the species. These criteria, however, often do *not* distinguish between enzymes which may exist in solution in the periplasm (if any constituents of the periplasm do indeed exist as aqueous-soluble macromolecules) or those "loosely" fixed to the membrane but shock-releasable. On the other hand any enzyme found to participate in accomplishing transport and also purifying with the vesicles is assumed *not* to be simply adsorbed to the membrane but to be functionally associated both as isolated and *in situ*.

type, approximately half the deoxyguanosine uptake activity is inhibited by deoxycytidine; a showdomycin-resistant mutant exhibits only half the deoxyguanosine uptake activity of the parent, with no inhibition by deoxycytidine. These authors suggested the existence of two deoxyguanosine uptake systems, one of which is deoxycytidine- and showdomycin-sensitive and presumably lost on mutation to showdomycin resistance. Though they tried to relate these two transport systems to the systems they had postulated for adenosine, such comparisons would be quite tenuous due to their own report of adenosine metabolism prior to uptake.

B. Pyrimidines

1. Pyrimidine Base Utilization

Conclusions concerning mechanisms of pyrimidine base uptake in bacteria – *Pyrimidines are taken up as uracil or orotate by a process of group translocation across the membrane involving the participation of uracil phosphoribosyltransferase and orotate phosphoribosyltransferase, respectively, and requiring PRPP. Cytosine must be deaminated to uracil prior to uptake. The deaminase is found in the periplasm. The work from which these conclusions are derived is discussed below.*

a. Uracil Uptake

The utilization of uracil and cytosine appears to be mediated by the uracil phosphoribosyltransferase.[5,36] The enzyme would act directly upon uracil and indirectly on cytosine (only after deamination to uracil). An involvement of the uracil phosphoribosyltransferase in uracil uptake is implied by the findings of Hochstadt-Ozer and Stadtman[5] who demonstrated stimulation of uracil uptake by PRPP in starved *E. coli* K_{12} and a surface localization of the uracil PRT'ase as evidenced by its release upon osmotic shock. The extent of release of uracil phosphoribosyltransferase into the shock fluid is within 10% of the extent to which uracil uptake is diminished in shocked cells. Working with *Salmonella,* Beck et al.,[36] isolated mutants for the uracil phosphoribosyltransferase and demonstrated the necessity of a functional uracil PRT'ase for uracil utilization. The strain lacking uracil PRT'ase was also found to be completely 5-fluorouracil resistant. Conversely, *E. coli* strain LW2, selected from its parent EA1 for 5-fluorouracil resistance, was unable to take up uracil (a 30-fold decrease over the parent) and lacked 97% of the parent's uracil PRT'ase activity (Bulman and Hochstadt, unpublished). Data obtained by Hochstadt and Bulman comparing uptake, enzyme activity, and ATP inhibition of uptake and uracil PRT'ase activity are shown in Table 4. The course of uracil uptake in the two *E. coli* strains is shown in Figure 8. Further indication of a periplasmic location of the uracil PRT'ase is provided by the finding that the usual techniques for membrane vesicles isolation[37,38] may completely remove the uracil PRT'ase from either *E. coli* or *S. typhimurium* membrane preparations. Transport activity is concomitantly lost (Bulman and Hochstadt, unpublished). In other *E. coli* strains (K_{12}), transport and enzyme activity are not necessarily destroyed as a consequence of vesicle preparation.[8] Selective release of periplasmic enzyme attributed to culture conditions in a given strain, as would be surmised in this case, has already been reported.[5]

The notion that uracil PRT'ase mediates uracil transport has been challenged by Grenson[39] who observed in yeast that starvation could result in subsequent uptake of small amounts of free uracil in a mutant lacking uracil PRT'ase and not exhibiting observable uracil uptake under normal growth conditions. The possibility that the *E. coli* system might, only appear to be uracil PRT'ase-dependent, as proposed by Grenson[39] for yeast, was investigated by Pierard et al.[40] These workers reported that in several *E. coli* uracil PRT'ase negative strains, uracil uptake is reduced to less than 1% of the wild-type and that pyrimidine starvation does not alter uracil uptake. They conclude that an absence of uracil uptake in such strains does not appear to be a consequence of a block in utilization. Thus, Grenson's criticism appears invalid at least with respect to *E. coli.*

Von Dippe et al.[41] also reject the involvement of uracil PRT'ase in the mechanism of uptake in *E. coli* B, citing as evidence the failure of Crawford et al.[42] to detect uracil PRT'ase activity. It is most unfortunate that Von Dippe et al.[41] rely on a report from another laboratory submitted 17 years before their own regarding the characteristics of the bacteria they are using presently, rather than assay enzyme activity in their own strains. The strain identified as *E. coli* B by Von Dippe et al. may indeed possess uracil PRT'ase and at least their attempts to measure it would seem to be of

TABLE 4

Uracil Phosphoribosyltransferase and Uracil Uptake in LW2* and EA1*

UMP formed, pmol/min/μl extract derived from 12μg cell dry weight

	LW2	EA1
Crude sonic extract	0.335	19.9
Osmotic shock fluid	0.71	20.9

Uracil taken up, pmol/min/12μg bacterial dry weight using starved *E. coli* cells.

Addition	Strain			
	LW2		EA1	
	unshocked	shocked	unshocked	shocked
None	0.0	0.0	0.15	0.0
PRPP, 5 m*M*	0.0	0.0	21.40	0.0
Ribose-1-P, 5 m*M*	0.0	0.0	9.40	0.0
ATP, 10 m*M*	0.0	0.0	7.20	0.0
PRPP, 5 m*M*, ATP, 10 m*M*	0.0	0.0	13.23	0.0
Ribose-1-P, 5 m*M*, ATP, 10 m*M*	0.0	0.0	6.25	0.0

*LW2 5-Fluorouracil resistant
*EA1 5-Fluorouracil sensitive

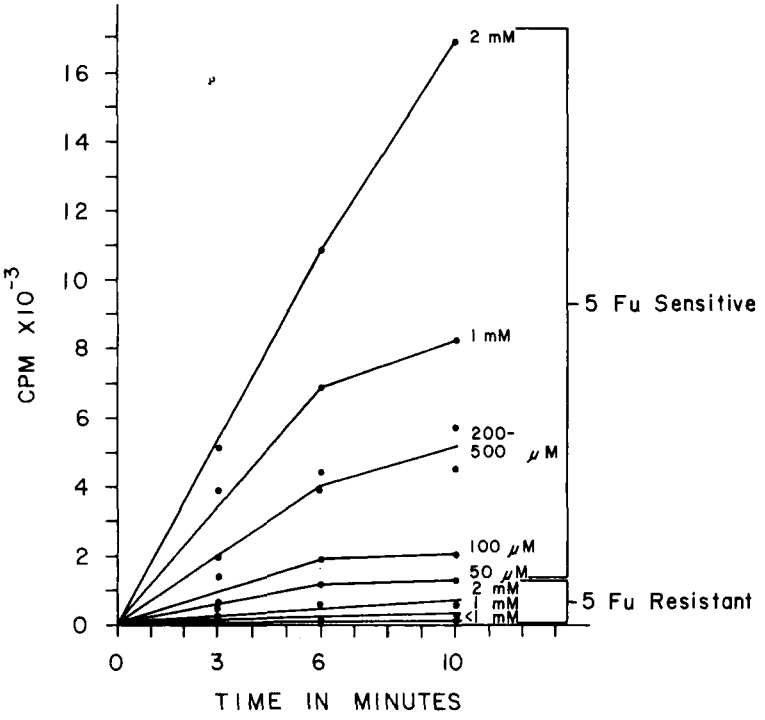

FIGURE 8. Uptake of uracil in a 5-fluoruracil sensitive strain and its 5-fluoruracil resistant mutant. Cells of each strain were grown overnight at 37°C in 100 ml of nutrient broth after loop inoculation, diluted to 80 to 100 Klett units (#66 filter) in fresh medium, and allowed to grow with moderate shaking for 2 additional hr at 37°C. The cells were harvested by centrifugation and washed four times with Med A salts.[5] Suspensions in Med A salts measuring 300 Klett units (#66 filter) were incubated for 5 min with 10 m*M* glucose (final) and 2-^{14}C-uracil was added at the final concentrations indicated. The reactions were terminated and uptake ascertained as described in Reference 5.

considerable importance in supporting the argument advanced.

As indicated in Table 4, 5-fluorouracil (FU) resistance in *E. coli* conferred by loss of 96 to 98% of uracil PRT'ase activity is accompanied by an inability to take up uracil. In the experiment of Table 4, uracil PRT'ase is completely recovered in shock fluid obtained from EA-1, unlike shocked K_{12} which retains a significant proportion of the enzyme and uptake activity is observed in whole cells after shock[5] and in the vesicle fraction after isolation.[8] Unshocked, starved FU sensitive cells (EA-1) take up little uracil without an energy source. With PRPP, ribose-1-P, or even ATP, considerable uptake is observed. Because ATP stimulates uptake, these starved cells are likely capable of utilizing some ATP (undoubtedly after its breakdown). Thus, the greatest barrier to ATP utilization by fresh cells is possibly its inaccessibility to the periplasm rather than a lack of periplasmic phosphorylases or phosphatases. The fact that ATP appears to stimulate rather than inhibit uracil uptake when it is the only energy source indicates it to be a much less potent negative effector of uracil than of adenine uptake (cf. Reference 5). In combination with PRPP or R-1-P, ATP at 10 m*M* does inhibit the full stimulatory effects of either of the sugar phosphates, suggesting that the activity observed in the presence of ATP is the sum of its stimulatory effects after metabolic conversion and its inhibitory effects as a regulatory effector. Ribose-1-P probably must also be metabolized in order to serve as an energy source (cf. Figures 3, 4, Reference 5). Starvation does not increase uracil uptake in the mutant LW2, an observation consistent with the results of Pierard et al., that further weakens any relation between Grenson's observations in yeast and the mechanism(s) operating in enteric bacteria. Though a more intensive and systematic investigation is required before any definitive statements can be made, the body of genetic, transport, and enzymatic evidence certainly favors the concept that uracil is taken up by the membrane-periplasm uracil phosphoribosyltransferase mediating a group translocation in much the same manner of adenine group translocation across the bacterial membrane.[4,5] More recently, and more compellingly, Finch and his co-workers in Australia have confirmed the group translocation role of uracil PRT'ase in uracil uptake by *E. coli*. These workers preloaded cells with uracil labeled with one radioactive isotope and then observed the specific radioactivity of cellular UMP immediately after the cells were exposed to uracil with a different radioactive label. They found that all endogenous UMP is formed only from exogenous uracil and not from intracellular uracil. In addition, the uracil PRT'ase was quite unstable upon purification and thus difficult to work with in purified extracts (L. Finch, personal communication). The possibility that UMP might be formed periplasmically and transported as UMP is an unlikely alternative. Addition of uridine and ATP in the presence of uridine kinase does not result in the uptake of the pyrimidine by vesicles when uracil PRT'ase is missing (Hochstadt and Radeř, unpublished).

Uracil uptake in isolated membrane vesicles of *E. coli* K_{12} can be regulated by ppGpp[8] to the extent that uridine nucleotide pool appeared to contract when measured by the uptake of labeled uridine. Uptake of uracil was completely inhibited by physiological concentrations of ppGpp present during amino acid control, although uptake of other bases was only partially shut down under these conditions.

b. Cytosine Uptake

Cytosine utilization in *E. coli* was examined by Hochstadt-Ozer and Stadtman,[5] the K_m for uptake being 65 μM. PRPP stimulates uptake but does not lead to saturation. It has been assumed that all the cytosine entering the periplasm is converted to uracil since a separate cytosine phosphoribosyltransferase has never been detected[5] and strains lacking UTP aminase (pyr G^-) cannot utilize cytosine as a precursor for CTP.[36,43] Even more convincing is the observation of resistance to 5-fluorocytosine only at the restrictive temperature in a temperature-sensitive 5-fluorouracil resistant mutant lacking functional uracil PRT'ase above 30°C.[36] Further, a cytosine deaminaseless pyrimidine auxotroph fails to grow on cytosine.[36] It thus seems likely that cytosine is utilized only after periplasmic deamination.

The K_m of 65 μM for overall cytosine uptake[5] is higher than the 43 μM K_m observed for uracil uptake in the same strain (Hochstadt, unpublished). Cytosine can be deaminated rapidly enough for meeting the entire nitrogen needs of *E. coli* at full growth rate.[36] The apparent K_m for cytosine uptake is only slightly greater than that for the transport of uracil transport, the immed-

TABLE 5

Uptake of Orotic Acid

Addition	Strain	
	EA (takes up uracil)	LW (does not take up uracil)
	Orotic acid taken up pmol/μg cells/min	
None	1.06	1.56
PRPP, 5 m*M*	1.68	2.41
PRPP, 5 m*M*, ATP 10 m*M*	0.71	1.05
PRPP, 5 m*M*, GTP 10 m*M*	0.61	0.91

iate putative precursor for the uptake system. This situation is different than the K_m(s) observed for the purine phosphorylase-phosphoribosyltransferase interaction suggested in purine uptake. Thus, "enzyme substrate passing" may only operate for nucleoside cleavage or deamination, and not base deamination. Regulatory data on the other hand, suggests that "enzyme cooperation" might, nevertheless, function with regard to cytosine utilization. The effects of ppGpp on "cytosine" uptake in vesicles from *E. coli* is an example of such regulatory effects in the utilization of cytosine.[8] At ppGpp concentrations that completely abrogate uracil uptake, cytosine uptake is inhibited by only 50%. Presuming a partially competitive type of relationship on the uracil PRT'ase (as has been seen for 5′-phosphoribosyl compounds on other PRT'ases tested,[3,8]) the PRT'ase affinity for the cytosine deaminase complexed uracil moiety being higher than for free uracil could explain why ppGpp inhibits cytosine uptake to a lesser extent than free uracil uptake.

Orotate uptake, though not extensively studied, may well involve a mediating phosphoribosyltransferase, because of the influence of PRPP and nucleotide effectors on utilization by whole cells (Table 5). Moreover, it is known from studies with mutants that the putative orotate enzyme is distinct from the uracil PRT'ase. However, orotate uptake could not be observed in vesicles due to removal of the uptake system during preparation (cf. Reference 37); these vesicles also lacked uracil uptake capacity. Whether a membrane-localized orotate PRT'ase activity in uptake exists and is distinct from the cellular enzyme active in *de novo* pyrimidine biosynthesis remains to be demonstrated.

The postulation of still another periplasmic and/or membrane-associated PRT'ase reemphasizes the need for investigation of the mechanism of PRPP synthesis or secretion and of accessibility to the surface PRT'ase. Thus far, only phosphopentomutase, which converts ribose-1-P (available from nucleoside cleavage) to ribose-5-P, the immediate precursor of PRPP, has been located in the periplasm.[29] However, evidence to be discussed below indicates that the ribose-1-P resulting from nucleoside cleavage in the periplasm is translocated in that very process. ATP secretion into or production within the periplasm would also be required for local PRPP synthesis. It seems more likely that PRPP is generated inside the cell and gains access to the PRT'ase transport systems from the inner side of the membrane, as is the case for PEP in the group translocation of certain sugars across the membrane.[44]

c. Thymine Uptake

Thymine uptake and utilization in *E. coli* and other bacteria has been studied by Munch-Petersen[33] and Kammen.[46] It was already known that *E. coli* does not use thymine unless required[47] and that thymineless strains deprived of exogenous thymine release significant amounts of deoxyribose into the medium.[48] Thymine can be utilized by wild-type strains, however, in the presence of exogenous deoxyadenosine.[49] Other deoxyribonucleosides will stimulate thymine uptake in wild-type *E. coli*, but deoxyadenosine seems to serve best.[33] Thymine requiring strains take up the base independent of deoxyribosyl donors, indicating that their mechanism of thymine uptake is likely to differ from that of the wild-type cells.[33] Reversion to thymine prototrophy also returns the strain to deoxyadenosine dependence of thymine uptake.[33] Thus, the two strains seem to differ not only in the ability to synthesize thymidine nucleotides, but also in their

deoxyriboside metabolism. Further, thymine requirers do not respond to exogenous thymidine by derepression of thymidine phosphorylase unless starved for thymine.[33] Conversely, thymidine phosphorylase is induced by thymidine in non-requirers.[47,50] Two revertants able to grow without thymine and again showing deoxyadenosine dependence of thymine uptake were still unresponsive to thymidine induction of thymidine phosphorylase and in this respect still resemble the thymine requirers. It was thus postulated by several laboratories that most isolated thymine requirers are likely to be double mutants.[33,48,51] Finally, the uptake of thymine requires a deoxyribosyl donor other than deoxyribose-1-P.[33] In order to ascertain the mechanism(s) which could account for the above observations, Munch-Petersen studied two reactions under a number of conditions:

1. thymine + deoxyribose-1-P → thymidine + P_i (catalyzed by thymidine phosphorylase (E.C. 2.4.2.4.).

2. $base_1$-deoxyriboside + $base_2$ → $base_2$-deoxyriboside + $base_1$ (catalyzed by trans-N-deoxyribosylases (E.C. 2.4.2.6.).

In reaction 2., deoxyadenosine and thymine were used as substrates. Munch-Petersen found that activities catalyzing both reactions are released upon osmotic shock. Moreover, thymidine phosphorylase activity increases upon release by osmotic shock treatment over the amount measurable in whole cells by as much as 12-fold.[33] The trans-N-deoxyribosylase activity, however, considerably diminishes upon release from the periplasm membrane.[33] A similar situation was later seen by Hochstadt-Ozer and Stadtman for adenine exchange into AMP catalyzed by the membrane localized adenine PRT'ase.[4] When this enzyme is removed from the membrane, the exchange reaction is catalyzed only by the membrane fragments containing the residual adenine PRT'ase and not by the solubilized PRT'ase.[4] This is also reminiscent of the hypoxanthine-guanine membrane PRT'ase and its uptake specificity, in which case the membrane constrains an enzyme in a conformation which can either restrict, broaden, or totally alter substrate specificity. In Munch-Petersen's experiments, significant activity for either of the two reactions remaining with cells after osmotic shock was only observed for the thymine-requiring strain. Neither activity was induced by growth on thymine itself, but required a deoxyribonucleoside and indicated the inducer includes a deoxyribosyl moiety.

Though it might seem most difficult to incorporate all of these observations into a model for thymine utilization, there is one point that does simplify the matter and does not require an explanation invoking double mutations. Since no mechanism for the direct biosynthesis of deoxyribose-1-P has ever been described, we must assume any deoxyribose or deoxyribose-P found in the cell or the medium can be presumed to have originated via UMP → dUMP → UdR → deoxyribose or deoxyribose-1-P + uracil. Thus, in cells unable to methylate dUMP to TMP, dUMP is degraded to a deoxyribosyl compound (which cannot be reutilized) and to uracil which is reutilized via uracil PRT'ase and PRPP. Thymine requiring cells would then be expected to have a pool of free deoxyribose-1-P. In such cells, thymidine phosphorylase appears fully induced when sonic extracts of the cells are assayed,[45] though the levels observable in intact cells can be only 10% of the enzyme activity in the sonic extract. Thus, either exogenous thymidine leading to endogenous deoxyribose-1-P or endogenous accumulations of dUMP (also leading to endogenous deoxyribose-1-P), could each serve to induce high levels of thymidine phosphorylase. Deoxyadenosine independence of thymidine uptake may simply reflect that deoxyuridine (almost as good as deoxyadenosine in potentiating thymine uptake) levels are elevated in cells unable to convert dUMP to TMP, and quite high when compared to the internal concentration of deoxynucleoside gaining access from exogenous sources. The membrane *trans*-N-deoxyribosylase can be envisioned to carry out a "group translocation" of an exchange type. Like other group translocations, exogenous thymine would enter the cell, and ultimately be converted to thymidine. However, no high energy phosphate would be driving the reaction as when PRPP or PEP drive the transport reactions in which they participate.[4,44] The reaction involving exogenous thymine and endogenous UdR would proceed autocatalytically ("turnstile" fashion). This could explain the high intracellular levels of thymidine phosphorylase.

Since the "intracellular TdR phosphorylase" is shock releasable, it may in actuality be membrane embedded and so forced into a conformation

precluding exposure to the surface. Although the trans-N-deoxyribosylase and the phosphorylase activities are not in constant ratio for each strain and growth condition, this does not preclude the same enzyme being responsible for the two activities. The membrane milieu would be expected to vary as would restraints placed on the protein leading to restrictions in its specificity. It is thus possible that pyrimidine phosphorylases in enteric bacteria act solely in a trans-N-ribosylating fashion across the membrane.

A recent report proposes the alternative to a trans-N-ribosylating function. Jensen et al.[51a] propose thymine utilization depends on an endogenous pool of deoxyribose-1-P, involves thymidine phosphorylase, but is independent of other deoxynucleosides. It is now apparent that enzymes of the periplasm and the membrane involved in initial nucleic acid precursor metabolism act differently than had originally been presumed. Nucleic acid precursors are processed into the nucleotide pools via a two compartment system in which periplasmic reactions are most significant. The role of transribosylations in utilization will be discussed further in the following section of pyrimidine nucleoside utilization.

2. Pyrimidine Nucleoside Uptake

Conclusions concerning the uptake of pyrimidine nucleosides into bacteria – *Uridine is cleaved to uracil and ribose-1-P in a group translocation reaction in which the ribose-1-P is released to the inner surface of the membrane while the uracil is released to the periplasm. The uracil is then taken up mediated by uracil phosphoribosyltransferase, an enzyme which is easily released from the membrane by osmotic shock procedures. The ribose-1-P accumulation in the first step is electron-transport stimulated. Cytidine is taken up by the two alternate pathways. One involves deamination as well as cleavage of the nucleoside and results in uracil and ribose-1-P as the intermediates actually transported across the membrane. The uracil accumulates at UMP. The second mechanism of cytidine uptake involves neither deamination nor cleavage, is feedback-inhibited by a number of related nucleic acid precursors, and is also electron-transport coupled. The experiments upon which these conclusions are based are herein presented and discussed or both.*

a. Uridine Utilization

The genetic analysis of a large series of mutants of *Salmonella typhimurium* deficient in one or more functions of nucleoside and nucleotide metabolism allowed Beck et al.[36] to piece together some of the elements of uridine utilization. They proposed that exogenous uridine can be utilized via one, two, or possibly three alternative pathways. One pathway requires the presence of uridine phosphorylase and uracil phosphoribosyltransferase. The growth rate on uridine of organisms lacking the phosphoribosyltransferase is only about one fourth that of the parent strain possessing the enzyme. Thus, it was concluded that approximately 75% of exogenously supplied uridine enters the cell via this route. Since both uracil PRT'ase[5,20] and uridine phosphorylase[23,29] are released to the shock fluid, and are thus presumed to reside in the periplasm or on the outer surface of the inner membrane, the most likely utilization mechanism is a two-step membrane-localized reaction similar to that described by Hochstadt-Ozer for adenosine uptake in *E. coli*.[6] A second pathway is presumed to function via uridine kinase and would involve direct phosphorylation of uridine. Attempts were made by Beck et al.[36] to determine whether this pathway could support growth at maximum rate if the uridine phosphorylase mutation were introduced into a strain lacking uracil PRT'ase, so that the uridine provided could not be shunted off to uracil. Full growth rate could not be restored. It was found that some uridine was broken down to uracil even when uridine phosphorylase was missing. Secondly, one of their strains which lacked both uridine kinase and uridine phosphorylase still grew on uridine. Thus, though this pathway is an obvious one (since uridine kinase has been identified), it may not function at all in the utilization of uridine, since there seems not to be any real dependence (80% normal growth rate observed) on it when alternate routes are blocked.

Of greater interest, however, is the function called "udc" by Beck et al.[36] Strains that are udc$^-$ are prevented from utilizing uridine, but can use uracil. This mutation was imposed on the uridine phosphorylaseless and uridine kinaseless strain and does not map in the uridine phosphorylase region of the chromosome. The udc locus may thus encode the reaction cleaving uridine to uracil and free ribose; further work is necessary to identify this function.

Several studies have dealt with the route of uridine utilization by uptake experiments rather than by genetic analysis. The first of these, from the laboratory of Peterson,[26] assumed all metabolism to be subsequent to uptake, so that uracil formation was taken as the measurement of uptake. The recently attained understanding of the periplasmic space as a real entity[52] and the identification of uridine phosphorylase as a periplasmic enzyme have invalidated this approach. Peterson et al. further assumed that material recovered in the filtrate had been excreted; this conception is to be viewed cautiously though it persists in more recent studies,[26,41,53] particularly since the method used to terminate reaction is dilution into ice-cold medium. Such a procedure is likely to cause a cold shock release of small molecules[35,63a] and should be replaced by washes with high salt solutions at ambient temperature[5,61,63a] to eliminate the introduction of artifactual results.

Von Dippe et al.[41] studied uridine uptake in *E. coli* B and in a showdomycin-resistant mutant derived from it. With showdomycin-treated cells of both strains, equal amounts of uracil and ribose-1-P are recovered from the medium, while with the untreated sensitive strain, much larger amounts of extracellular uracil than sugar phosphate are accumulated. Though it may be the case that the uridine analog showdomycin (3-β-D-ribofuranosylmaleimide[54]) blocks uridine utilization, the quantitative basis of these experiments must be questioned because of the possibility of cold shock under the conditions employed for terminating reaction.

Cell-free extracts from shocked and unshocked cells and shock fluid were used to evaluate the effect of showdomycin on soluble uridine phosphorylase. No inhibitory effect was observed. Two possibilities thus exist: either showdomycin must be metabolized by some membrane component disrupted during extract preparation, or its site of action on the membrane is disrupted during extract preparation. The latter possibility could relate to the as yet undefined function controlled by the udc locus described by Beck et al.[36]

Bearing on the resolution of these results in terms of a model of uridine transport is the recent work of Hochstadt and Rader[20,21,55] and to be published in which uridine transport was studied in isolated vesicles of *E. coli* and *S. typhimurium*. In addition to study in other strains, uridine uptake was assayed in *E. coli* LW2 which lacks uracil PRT'ase and is incapable of uracil uptake. It should be remembered from a previous section that vesicle preparation of EA1, the uracil PRT'ase containing parent of LW2, also- lost the uracil PRT'ase and uptake function upon vesicle preparation. Surprisingly, however, vesicles of both strains are able to take up counts from labeled uridine.[20,21,55] It was soon observed, however, that uptake of counts only occurs if uniformly labeled ^{14}C-uridine is used and little or no uptake is observed when 2-^{14}C-uridine is substrate. The vesicle contents were shown to contain ribose-1-P; the medium surrounding the vesicles, uracil. Most surprisingly, however, ribose-1-P, and ribose are neither substrates for uptake nor cause significant inhibition at concentrations similar to the uridine used while ribose-5-P did inhibit (Rader and Hochstadt to be published). Thus, a *trans*-membrane phosphorylase is a likely mechanism for uridine transport in which uridine interacts with the membrane, releasing ribose-1-P inside the cell and uracil to the outside. An alternative hypothesis is that uridine is taken up intact, cleaved intravesicularly, and the vesicles retain ribose-1-P while leaking uracil back to the medium. The uracil is taken up predominantly by uracil PRT'ase when present and by some other process which may be unrelated to uracil PRT'ase (cf. Reference 36). The putative *trans*-membrane phosphorylase function, which is specific for certain nucleosides (adenosine is even more inhibitory or competitive than uridine itself in the uptake assay for the ribose from uridine), is an electron-transport-linked transport system,[20,21,55] and is stimulated by L- and D-lactate and succinate to varying degrees depending upon the medium in which the cells were grown. Such electron-transport coupling of substrate transport has been well studied for a number of sugars and almost all amino acids (cf. Reference 56), but this represents the first implication of such a mechanism for nucleic acid precursor transport.

b. Cytidine Utilization

Cytidine and deoxycytidine utilization have been studied in a number of laboratories using a number of normal and mutant strains of *E. coli* and *S. typhimurium*.[20,21,24,26,32,41,53,55,57] The pathway of cytidine utilization has been proposed to involve either direct phosphorylation by uridine (and cytidine) kinase (the same

TABLE 6
Shock Fluid: Cytidine Metabolism

(Chromatographic results after exposure of shock fluid to ^{14}C cytidine)

	No additives	+ATP	+GTP
	cpm	*cmp*	*cpm*
Cytosine	147	11	18
Cytidine	157	107	160
Uracil	1,607	1,381	1,679
Uridine	227	527	324
CMP	37	181	161
UMP	375	994	751

enzyme) or deamination and cleavage to uridine and uracil respectively.[36] It is, however, difficult to reconcile a direct phosphorylation mechanism since mutants lacking the kinase and the uracil PRT'ase, but possessing cytidine deaminase, can utilize deoxycytidine.[36] There is thus a route from cytidine to uracil nucleotides that, although not requiring cytidine deaminase, cytosine deaminase, uridine kinase, or uridine phosphorylase, does require uracil PRT'ase. Utilization of cytosine *does*, however, depend completely on cytosine deaminase.[36] Further, the udc function described by Beck et al. is necessary for cytidine utilization as well as uridine utilization.

Though a mechanism involving *trans*-N-ribosylase has been discussed for thymine and a related mechanism seems to be involved in the internal ribose-1-P accumulation (resulting from vesicle uridine interaction while the uracil is released), an additional mechanism merits consideration: a nucleoside transphorphorylase in which the transesterification: $base_1$ ribose + $base_2$ ribose-P → $base_2$ ribose + $base_1$ ribose-P (e.g., GMP + adenosine → guanosine + AMP) might occur across the membrane. Nucleosides would always be in the periplasm, and nucleotides always in the cell. This is suggested from results of studies with vesicles:[20,21,55] not all cytidine utilization seems to occur via uridine pathways after deamination. Moreover, cytidine and uridine uptake are unequal in magnitude and differentially stimulated by electron-transport effectors. The ribose moiety, whether from U-^{14}C-uridine or U-^{14}C-cytidine, is taken up even more efficiently than the nucleosides or bases. Thus, cytidine uptake in isolated vesicles is the summation of the uptake of the ribosyl moiety from cytidine deaminated to uridine as well as the cytidine utilized intact. The properties of the uptake, though involving two mechanisms, have been preliminarily reported by Hochstadt[20] and are detailed as follows.

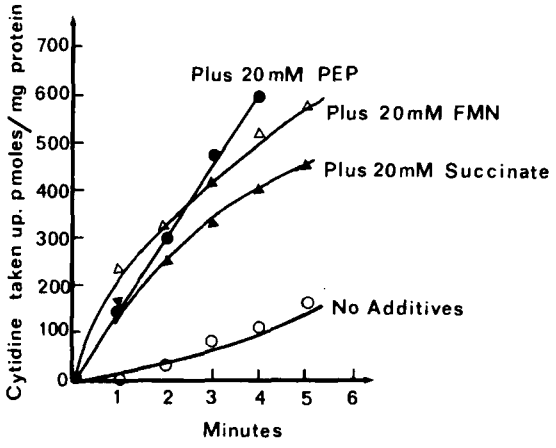

FIGURE 9. Uptake of cytidine into membrane vesicles from *S. typhimurium* LT-2. Membrane vesicles were prepared and assayed as described in Reference 4. Uptake of U-^{14}C-cytidine 345 mCi/mmol (New England Nuclear) was measured at final concentration of 30 μM. Effectors listed at concentrations indicated were added immediately before adding cytidine to initiate the reaction. Buffer consisted of 1 mM Mg^{2+} + 0.1 M (K^+) PO_4, pH 7.5. Reactions were performed at 31°C.

Isolated membrane vesicles having substantially lost enzyme constituents of the periplasmic space were prepared by the lysozyme-EDTA method.[4,37] The shock fluid, however, contains cytosine and cytidine deaminases, uridine phosphorylase, (as seen in Table 6) and uracil phosphoribosyltransferase. These activities are detected after incubating shock fluid, cytidine, and any additions indicated, at 37°C for 15 min, and chromotographing an aliquot of the reaction mixture on cellulose thin layers.

Isolated membrane vesicles best transport cytidine of all pyrimidine bases and nucleosides. The cytidine uptake seems to occur by a mechanism unrelated to those previously indicated for uptake of purine nucleosides and bases, or of uracil or cytosine. It does, however, resemble transport of amino acids, K^+, certain sugars, and that just suggested for uridine in that uptake seems to be electron-transport system (ETS) linked.[56] Figure 9 shows the uptake of cytidine in the presence and absence of certain energy sources. PEP also stimulates uptake, presumably after its conversion to L-lactate which is also an effective energy source.

The vesicles contain less than 2% of the cellular RNA, 3% of the cellular DNA, 0.1% of several cytoplasmic enzyme markers (cf. Reference 44) and about 10% of the total diaminopimelic acid, but are made with limiting EDTA and limiting

TABLE 7

Cytidine Uptake in Isolated Membrane Vesicles

Addition	Concentration	Activity, p mol/mg/min vesicle protein
None	–	6.0
$MgSO_4$	5 m*M*	58.0
KPO_4, $MgSO_4$	65 m*M*, 5 m*M*	106.0
NaSuccinate, KPO_4, $MgSO_4$	20 m*M*, 65 m*M*, 5 m*M*	178.2

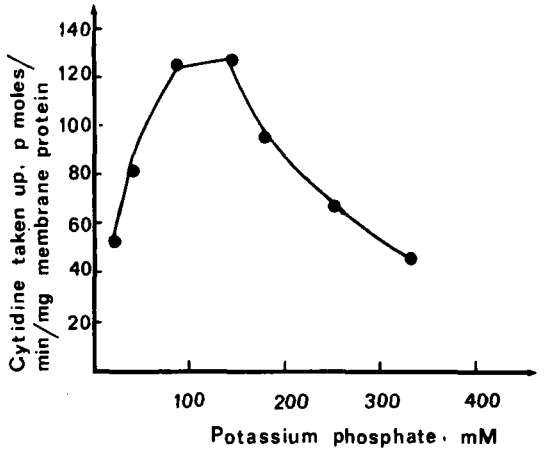

FIGURE 10. Effect of potassium phosphate concentrations on cytidine uptake. Experiment was performed as in Figure 9 with the exception that K^+ succinate, 20 mM (final), was added and potassium phosphate concentration was varied as indicated. The reaction was carried out for three min and the results are plotted in terms of uptake per minute.

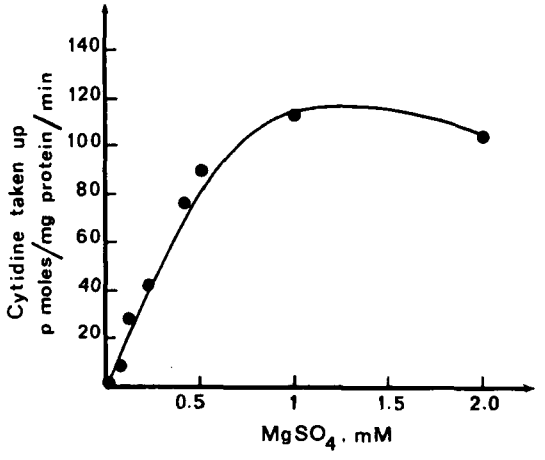

FIGURE 11. Effect of magnesium concentration on cytidine uptake. The experiment was performed as in Figure 10 with $MgSO_4$ varied while the potassium phosphate was held constant at 0.1 m*M*.

homogenization in order to retain ETS components. Table 7 shows the essential requirements for the uptake system while Figures 10 and 11 indicate the dependencies on (PO_4) and Mg^{2+}, respectively. The Mg^{2+} requirement is further emphasized by a marked sensitivity of transport to 8-hydroxyquinoline concentration (Figure 12). Increases in succinate concentration do not lead to uptake at constant rate; succinate is nonsaturable with respect to concentration. The cytidine transport system is relatively independent of external pH changes (Figure 13). The temperature optimum for 45 sec incubation is higher than for 2 min incubation (Figure 14), and rapid leakage is similar to other unphosphorylated substrates, such as amino acids (cf. Reference 44). Cytidine uptake is inhibited by the sulfhydryl alkylating agent (N-ethylmaleimide) (Figure 15), presumably by modification of the carrier, and energy uncoupling with CCCP and 2,4-dinitrophenol (Figures 16 and 17) were also observed. Potassium arsenate is not inhibitory while sodium azide, potassium cyanide, and sodium bisulfite depress uptake (Figure 18). These findings support the notion that oxidative-phosphorylation is not required for transport. The measured K_m for uptake is 16 μM.

The concentration of material taken up reached 80 to 100-fold relative to the extravesicular concentration, as determined by measurement of inulin and urea space. Several electron donors could serve as energy source and when added, in combination the electron donors are more stimulatory than individual donors, indicating that several different dehydrogenases might be coupled to energize the carrier system (Tables 8 and 9). When allosteric or regulatory inhibitors (e.g. 5′ nucleotides) are examined for their effect on cytidine uptake, uridine, CMP, and UMP are found to be inhibitory (Figure 19), but CTP, cytosine, and uracil do not inhibit uptake. The absence of inhibition by CTP is in contrast to the purine

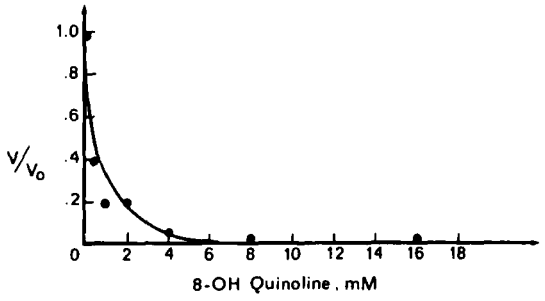

FIGURE 12. The effect of 8-hydroxyquinoline on cytidine uptake. The experiment was performed as in previous experiments. The membrane vesicles, 322 μg/determination, were preincubated with 8-hydroxyquinoline for 5 min prior to addition of succinate or cytidine. The results are plotted as the fraction of uptake activity observed after the three-minute incubation in the presence of 8-hydroxyquinoline. The rate observed in this experiment for the uninhibited control was 170 pmol/min/mg membrane vesicles.

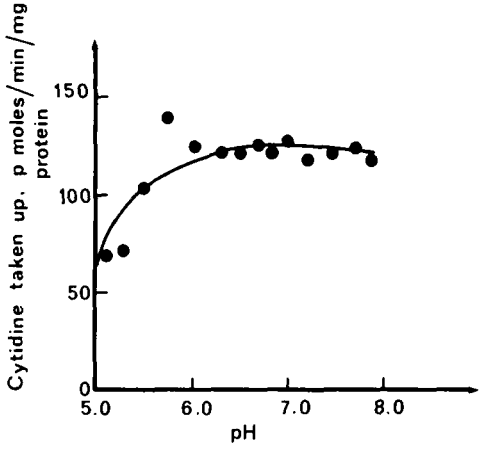

FIGURE 13. Effect of pH on cytidine uptake by isolated vesicles. The experiment was performed as for the previous figures with the following exception. Aliquots of membrane vesicles were collected from the 0.1 *M* potassium phosphate buffer pH 7.5 in which they were stored, washed once in 0.1 *M* potassium phosphate buffer at the pH indicated, and resuspended at their original volume in the new buffers. The membrane samples were then used as before. The reaction mixture (100 μl final) contained 100 μg membrane protein, three nmol U-^{14}C-cytidine, 10 μmol potassium phosphate, 0.1 μmol $MgSO_4$ and 2 μmol of K^+ succinate.

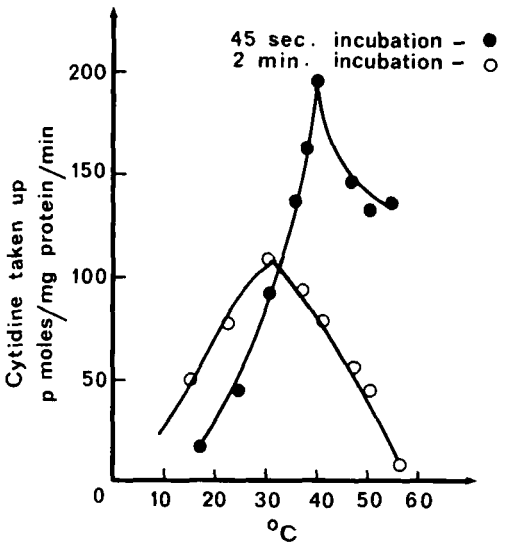

FIGURE 14. Temperature dependence of uptake and retention of radioactive material after exposure to U-^{14}C-cytidine for two time intervals. Membrane vesicles were incubated for 45 sec or 2 min with ^{14}C-cytidine after exposure to succinate, PO_4, $MgSO_4$ as in previous experiments at the temperatures indicated.

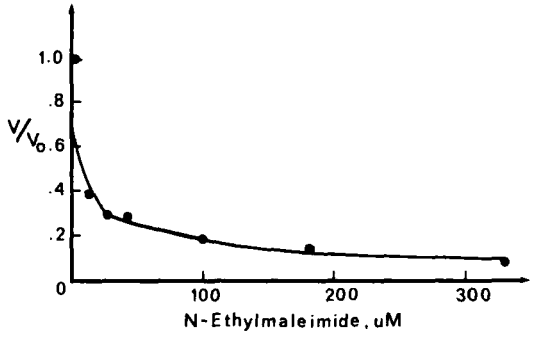

FIGURE 15. Effect of N-ethylmaleimide on cytidine uptake in *S. typhimurium* vesicles. The experiment was performed as in Figure 12.

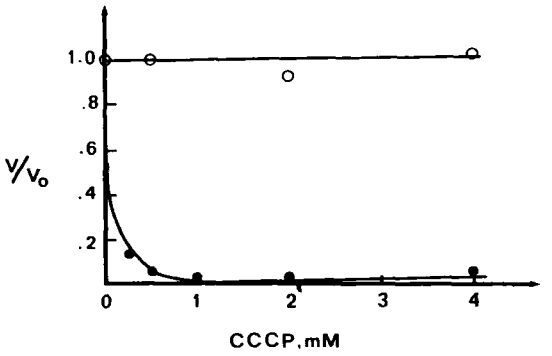

FIGURE 16. Effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on vesicle uptake of cytidine. The experiment was performed as in Figure 12. The results obtained from CCCP addition in ethanol at the concentrations indicated: (●); the upper curve gives the ethanol effects in the absence of CCCP: (0).

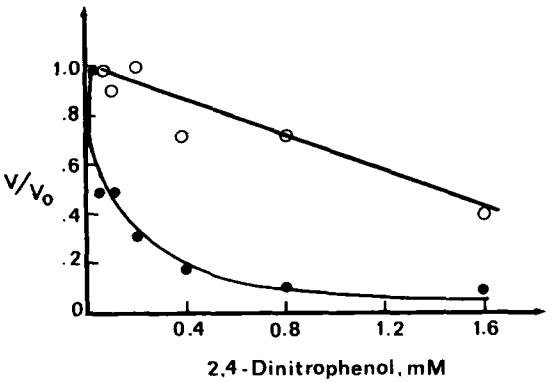

FIGURE 17. The effect of 2,4-dinitrophenol (DNP) on cytidine uptake. The experiment was as in Figure 12. The open circles describe the effects of the ethanol used, for obtaining each of the DNP concentrations.

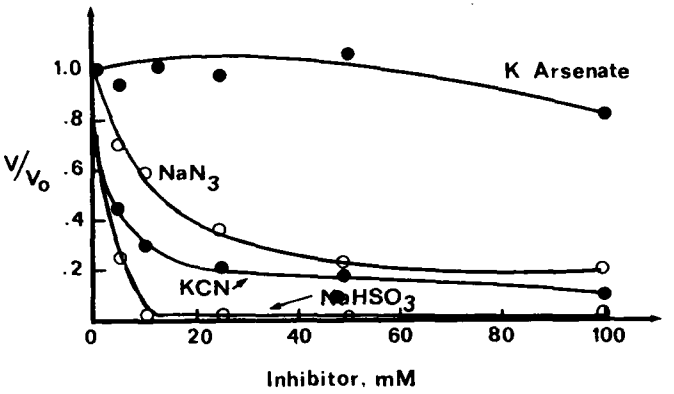

FIGURE 18. The effects of metabolic inhibitors on cytidine uptake into membrane vesicles. The experimental details and representation of results are as in Figure 12.

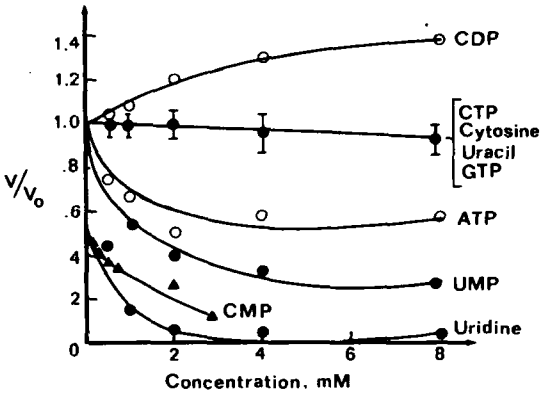

FIGURE 19. Effect of nucleic acid precursors on uptake activity for cytidine by isolated vesicles of *S. typhimurium* LT-2. Experimental details are identical to those in the experiment in Figure 12 with the exception that the compounds indicated were tested. The values for CTP, cytosine, uracil, and GTP were virtually identical. For clarity a single value is shown; the extremes of all values obtained are indicated for each concentration tested.

TABLE 8

Effectors of Cytidine Uptake

Addition, final concentration			Uptake, p mol/min/mg vesicle protein
$MgSO_4$ and KPO_4 only			101
$MgSO_4$ and KPO_4 plus:	Ascorbate	8 m*M*	96
	Citrate	8 m*M*	93
	CTP	4 m*M*	99
	Glucose	20 m*M*	98
	Glycerol	20 m*M*	100
	Malate	8 m*M*	108
	a methylglucoside	20 m*M*	97
	NADH	2 m*M*	90
	NADPH	4 m*M*	88
	PRPP	5 m*M*	90
	FMN	8 m*M*	162
	D-lactate	10 m*M*	144
	L-lactate	10 m*M*	127
	PEP	80 m*M*	150
	Succinate	20 m*M*	180
	ATP	2 m*M*	33
	GTP	8 m*M*	50
	FAD	8 m*M*	68
	Fumarate	8 m*M*	76
	Ribose-1-P	5 m*M*	69

TABLE 9

Cytidine Uptake in Isolated Membrane Vesicles

Addition	Concentration	Activity, p mol/min/mg vesicle protein
NaSuccinate, KPO_4, $MgSO_4$	20 m*M*, 65 m*M*, 5 m*M*	178.2
FMN, KPO_4, $MgSO_4$	20 m*M*, 65 m*M*, 5 m*M*	120.8
PEP, KPO_4, $MgSO_4$	20 m*M*, 65 m*M*, 5 m*M*	110.4
PEP, FMN, KPO_4, $MgSO_4$	20 m*M*, 20 m*M*, 65 m*M*, 5 m*M*	160.2
PEP, succinate, KPO_4, $MgSO_4$	20 m*M*, 20 m*M*, 65 m*M*, 5 m*M*	162.0
FMN, succinate, KPO_4, $MgSO_4$	20 m*M*, 20 m*M*, 65 m*M*, 5 m*M*	152.0
FMN, succinate, PEP, KPO_4, $MgSO_4$	20 m*M*, 20 m*M*, 20 m*M*, 65 m*M*, 5 m*M*	192.0
$MgSO_4$	5 m*M*	58.0
None	–	6.0

uptake situation in which the conjugate nucleoside triphosphate is always a potent inhibitor. A summary of our observations with this system can be found in Table 10.

In conclusion, cytidine uptake can be mediated by systems resembling at least in part classical active transport requiring electron transport system energy coupling. The failure to find a unique electron donor, rather than being novel, suggests a review of data from other laboratories. Bhattacharyya et al.[59] found that several electron donors stimulate valinomycin mediated K^+ uptake. Recently, Thompson and MacLeod[60] showed that a marine pseudomonad also has rather nonspecific requirements for dehydrogenase-linked energy sources to stimulate amino acid transport. Kaback's conclusions that a single donor is primary are to be questioned, since a mutant lacking the D-lactate dehydrogenase is capable of amino acid uptake in whole cells through coupling via the membrane succinic dehydrogenase.[61] Moreover, we have tested proline, serine, glutamate, and threonine uptake and obtained similar results: membranes carefully prepared to remove wall and

TABLE 10

Cytidine Uptake in *S. typhimurium* LT-2 DAP-3 is:

1. Concentrates > 60-fold.
2. Requires Mg, KPO_4: optima 1–5 m*M*, 75 to 140 m*M*, respectively.
3. Stimulated by succinate, FMN, D-lactate, PEP, L-lactate, CDP.
4. Inhibited by PRPP, R1P, ATP, 2,4 DNP, CCCP, NaF, N-EM, 8-OH quinoline, $NaHSO_3$, KCN, CMP, Uridine, UMP.
5. Unaffected by ascorbate, citrate, fumarate,* glucose, a-Methyl-glucoside, FAD,* glycerol, malate, NADH, NADPH, CTP, K-Arsenate cytosine, uracil, GTP.
6. Has flat pH optimum in KPO_4 between pH 6 to 8.
7. Has temperature optimum of 30° at 2 min, of 35° at 45 sec high temperatures okay 45 sec little retention 2 min.
8. $I_{0.5}$ for following compounds are DNP: 100 μM; CCCP: 40 μM; NaF: 10 m*M*; N-em: 4 μM; 8-OH quinoline: 300 μM; $NaHSO_3$; 2.5 m*M*; NaN_3: 14 m*M*; KCN: 4 m*M*; CMP: 100 μM; uridine: 100 μM; UMP: 1 m*M*; ATP: 10 m*M*.

*Somewhat inhibitory to base level.

cytoplasmic components but not overly homogenized or extensively treated with EDTA show many coupling sites for transport. Vigorous homogenization does seem to remove or inactivate all but one dehydrogenase coupling enzyme, which varies according to strain, growth medium, and even culture density at harvest. The conclusion on the part of Hong and Kaback[61] that the tightest bound activity is also the only important one conflicts with the results of Simoni and Shallenberger[62] and of Rosen (B. Rosen, personal communication) who have both obtained mutants that are able to transport amino acids if lacking D-LDH, but unable to do so when the membrane ATP'ase is lacking. Rosen has now been able to partially restore transport activity in the presence of DCCD, a known effector of the membrane ATP'ase. Multiple electron donors acting *in situ* all linked in some way to an ATP'ase could explain these findings.

c. Deoxycytidine Utilization

In *E. coli*, counterselection using deoxycytidine in the presence of penicillin results in the isolation of cytidine deaminase negative mutants.[24] Thus, deoxycytidine is not a substrate for uridine/cytidine kinase and deoxycytidine must be deaminated. This is also true for *S. typhimurium*, since Beck et al.[36] found deamination to be the first essential step to utilization. Several studies purport to follow deoxycytidine uptake per se[32,57] despite prior work showing that deamination is essential,[24,36] and even older studies localize cytidine deaminase in the periplasm.[23] These reports must instead represent uptake of UdR, uridine or uracil, or a combination thereof. Consequently, a postulation of multiple systems each with particular characteristics cannot be considered unequivocal and is more likely attributed to one or more of the uptake systems for the other intermediates. Komatsu and Tanaka[32,57] have described what they refer to as two deoxycytidine uptake systems; their evidence being the observation that a showdomycin resistant *E. coli* mutant still transports deoxycytidine, but at reduced rate and with no loss in efficiency in the presence of showdomycin. This is termed the showdomycin-resistant system. In the (sensitive) wild-type, cytidine uptake can be completely inhibited by showdomycin, and this is taken to indicate a second, showdomycin-sensitive, system. A more logical explanation of the findings, it would seem, is the postulation of a single system which loses all ability to interact with showdomycin and deoxycytidine upon mutation to resistance. As described by Komatsu and Tanaka, the systems are driven by electron donors and may be related to or partially interdigitated with the electron-transport coupled system for the ribose moiety of uridine and cytidine described by Hochstadt and Rader.[20,21,55] There are many indications that though uridine and cytidine nucleoside transport may share common pathway(s), such pathway(s) differ from pathways for purine nucleosides.

d. Thymidine Utilization

Thymidine seems not to be taken up well into vesicles or intact cells of strains capable of *de novo*

thymidine nucleotide synthesis.[4,5] The utilization of both thymidine and deoxyuridine seems to depend on functional thymidine kinase.[36] More extensive work on uptake of this important compound seems to be lacking, but merits considerable attention in the future.

C. Conclusions Concerning Bacterial Systems

Mechanisms for free base uptake have gained considerable understanding in recent years, while uptake mechanisms for nucleosides, particularly pyrimidine nucleosides, remained ill-defined. Two very important and broad concepts are, however, apparent. (1) The importance of the periplasm cannot be overemphasized in the nature and variety of reactions comprising the initial processing of nucleic acid precursors in enteric bacteria. (2) The relationship of the periplasmic or membrane enzymes to each other and to the precursor processing which occurs in the periplasm allows cooperation and exchange across the membrane in a variety of reactions. These reactions cannot proceed in the same way when the enzymes are liberated into solution and no longer aligned with one another. Such reactions involving membrane-localized enzymes are already implicated in inosine, adenosine, and thymine uptake, as well as in the possible membrane transphosphorylation that occurs in the formation of internal ribose-1-P from nucleosides. This leads to an appreciation of membranes not only as barriers, but equally as "assembly lines" for efficiency in certain biosynthetic pathways.

Furthermore, the use of ice-cold or hypotonic washes to define what metabolism has gone on in the cells and what products have been extruded to the medium must be viewed with extreme caution. The compartment of the periplasm and even release of cellular pools of small molecules occur under these conditions (cf. Reference 62a). Finally, knowledge is accruing that implicates periplasmic or exogenous precursors as more directly accessible than the cytoplasmic pool for certain macromolecular biosynthesis (cf. References 45, 63). In conclusion, the membrane and the periplasm are most important in coordinating many cellular activities, and many studies are contributing to the field, however, earlier presumptions excluding consideration of periplasm and shock-release phenomena must not be allowed to continue to obscure new emerging concepts.

III. UTILIZATION OF NUCLEIC ACID PRECURSORS IN ANIMAL CELLS

A. Purines

1. Purine Base Utilization

Conclusions concerning the uptake of purine bases in animal cells – *There seems to be a great diversity of mechanisms of purine uptake in animal cells. The involvement of phosphoribosyltransferases seems more apparent in tissue culture lines than in other cells studied but has only been clearly demonstrated for one substrate in one cell line (hypoxanthine uptake in Balb/c 3T3) using a subcellular isolated vesicle system. The details of work with several types of animal cells and established cell lines and the variety of uptake phenomena observed for purine utilization is presented in the section below.*

a. Adenine Uptake

Rabbit polymorphonuclear (PMN) leukocytes have been employed extensively by Berlin and his co-workers to study uptake of adenine and other nucleic acid precursors.[64-69] The rabbit PMN adenine uptake systems differ from two previously studied experimental systems for adenine uptake in animal cells. Erythrocyte adenine uptake is presumed to be a facilitated diffusion system (saturable but nonconcentrative and not energy-driven),[70] while adenine uptake into Ehrlich ascites cells is presumed to be unmediated passive diffusion (directly proportional to all external concentrations, i.e., nonsaturable).[71] PMN cell adenine uptake, as studied by Hawkins and Berlin,[64] has the following characteristics. Adenine is concentrated within the cells over 90-fold relative to the concentration in the external medium; radioactivity recovered from the acid-soluble material closely approximates total radioactivity taken up, indicating that little, if any nucleic acid synthesis, occurs during the experiment; nucleotides, particularly ATP, account for at least 97% of the radioactivity recovered; adenine accounts for less than 1%. The ratio between adenine, adenosine, AMP, ADP, and ATP does not change with time. Counterflow of free adenine does not occur, further indicating the absence of a free adenine pool. (Counterflow is the recapture to the medium of labeled adenine that has been previously concentrated internally by the uptake system, upon incubation with a high exogenous concentration of unlabeled adenine.) Adenine

label leakage could, however, occur subsequent to cell preloading at high adenine concentrations (conditions under which the transport system would not be operating to concentrate the adenine). This indicates that efflux impermeability is not the explanation of the previous result, but that adenine label in forms different from free adenine exists under the first of the two conditions. By this method, it was determined that entry even at the high adenine concentrations is not unmediated, but is controlled by a second transport carrier system with low affinity but high velocity. This latter system is of unknown significance, however, since its contribution would be negligible at physiological adenine concentrations, it has a $K_m = 100$ m*M* and a V_{max} equal to 13,700 pmol/45 sec/10^6 cells and does result in free adenine accumulation. The transport system operating at physiological adenine concentrations has a K_m equal to 7.0 μM and a V_{max} equal to 5.7 pmol/45 sec/10^6 cells and does not result in free adenine accumulation. The low K_m system is inhibitable by a variety of free purine bases but not by adenosine, uric acid, cytosine, or AMP. The failure of adenosine to compete or inhibit suggests the relationship of adenosine to adenine uptake differs from the relationship in bacteria.[6] The failure of AMP to inhibit might at first glance suggest that the mechanism does not involve adenine phosphoribosyltransferase or at least not in quite the same way as it is involved in bacteria (with adenine PRT'ase on the outside of the membrane). Findings to be discussed, however, might suggest that in animal cells the AMP was very rapidly degraded to adenosine by surface 5′ nucleotidases, and that by the time the experiment was actually begun, only adenosine, a noncompetitor, and not AMP, a product of a possibly involved PRT'ase, was present to compete.[72]

Berlin[65] suggests that AMP synthesis from adenine proceeds subsequent to rather than concomitant with transport, because transport is the rate-limiting step and certain inhibitors of the adenine phosphoribosyltransferase in extracts are not inhibitors of the transport-phosphoribosylation process in whole cells.[65] Similarly in the bacteria PEP phosphotransferase reaction, more highly specified conditions are required to effect vectorial phosphorylation (with the transport step rate-limiting) than to effect nonvectorial phosphorylation (catalysis without transport, which proceeds at faster rate). The two processes are performed in a single step only when the components of the transport system are in proper relation to one another in the membrane.[73] A similar situation has been noted for adenine transport in bacteria.[4]

Thus, a two-step reaction would be obviated if a dual role of membrane-bound enzyme carrying out both transport and catalysis were to be demonstrated. Berlin's finding that certain inhibitors of the enzyme do not inhibit transport could be explained by a protective effect of the membrane milieu that is eliminated on solubilization. This has been observed for adenine and other purine transport systems in isolated vesicles.[4,8]

These criticisms of the precise role of the membrane in the mechanism notwithstanding, Tsan and Berlin have made a most important contribution in the areas of membrane mosaicism and fluidity of membrane matrix components in their studies on correlations between transport and phagocytosis. After as much as 50% of the surface membrane has been invaginated in the process of phagocytosing polyvinyltoluene beads, no loss in adenine transport activity (both adenine systems and three other substrate systems were assayed) could be detected. It was shown that synthesis of new transport carriers for the surface concomitant with phagocytosis was not responsible for maintenance of identical transport kinetics. Para-chloromercuribenzoate at 5 μM causes 90% of inhibition lysine transport, but only minimally effects purine transport and phagocytosis. After excess pCMB is removed, lysine uptake is still inhibited. If new carriers were responsible for the maintenance of adenine transport while the membrane is invaginated, then new lysine carriers should also be generated and so restore lysine transport activity. The observation that lysine transport remains at the inhibited level indicates that either the portion of the membrane participating in transport does not invaginate or, as the membrane invaginates, transport systems are mobilized and relocate to noninvaginating portions where they can still function in uptake. The latter hypothesis seems the more likely based upon experiments investigating the "capping" phenomenon of plant-lectin interaction with cell surfaces.[74-76]

Though adenine transport can be readily demonstrated in whole cells from a variety of cell lines, adenine uptake into vesicles after membrane

TABLE 11

Comparative Uptake of Nucleic Acid Precursors by Five Cell Lines

Substrate	Cell line: HeLa cells: growing culture	HeLa nongrowing culture	Mouse 3T3 near confluent culture	CHO V79	BHK_{21} C_{13}
	Transport in pmol/min/10^6 cells				
Adenine	26.05	> 9.05	5.5	70	112
Adenosine	>16.3	9.36	24.0	188	88
Cytosine	*negligible*	*negligible*	4.5	0	22
Guanosine	> 9.1	6.03	1.6	nt	56*
Hypoxanthine	>54.1	23.6	18.0	70	100
Inosine	> 9.6	> 9.6	2.1	65	0
Thymidine	2.8	nt	0.242	nt	0
Uracil	negligible	negligible	4.1	0	65
Uridine	31.0	> 4.56	6.2	68	2.6
Xanthine	negligible	negligible	1.7	nt	nt
Xanthosine	nt**	nt	1.6	nt	nt

*Guanine
**not tested

isolation by the technique developed by Wallach and Kamat[79] has been much more difficult to demonstrate. Baby hamster kidney cells, however, if grown on casein hydrolysate medium and especially if the vesicles prepared are stored at -70°C for a period of time (weeks or months), do repeatedly produce batches which retain stable adenine uptake ability. The details of the experiments with such active batches of BHK cells that have been obtained in this laboratory are as follows.

Cells are grown to confluence or subconfluence (the medium being changed every two to three days), scraped from the roller bottles, washed in phosphate buffered saline and resuspended twice, and fractionated to obtain a membrane vesicle preparation free of other cytoplasmic components according to the procedure of Wallach and Kamat.[79] The Wallach and Kamat procedure employs nitrogen cavitation and differential centrifugation to homogenize and separate surface and endoplasmic membrane vesicles from other cell components. Finally, the surface and internal membrane vesicles are separated from each other by gradient or barrier centrifugation techniques, and the purity of the fractions is monitored by enzymatic markers.[81-85] Transport and nucleic acid precursor metabolic pathway enzyme assays are as previously employed[3-6] with the exception that: (1) smaller pore-size nitrocellulose filters are employed to retain the smaller membrane vesicles obtained by the nitrogen cavitation procedure, and (2) higher ionic strength (0.8 M NaCl) washes are used to terminate reactions as a means of control for the mammalian cell membrane's greater tendency to leak the transported substrates during the wash.

The first experiments of the series were aimed at determining the feasibility of transport assays in suspension, after scraping from glass, and for cells maintained at 4°C for several hours (as might be done to test various effectors – "resting cells".[4,5] We also wished to compare the uptake activity of cells in suspension with subcellular vesicle activities. The results of these tests are shown in Tables 11 and 12. As can be seen in the tables, there was great variability in rates; BHK 21 line gives the highest rates for a monolayer culture tested in suspension. Thus, the BHK/C_{13} line was chosen to develop our initial membrane vesicle transport system. Microsomal fractions (containing plasma membrane vesicles) were prepared[85] and tested for their ability to take up nucleic acid precursors. As can be seen in Table 13, initial rates are calculated in uptake normalized to 10^6 cells. Though the rates are considerably lower than observed in intact cells, they are quite significant and constitute a greater proportion of the whole

TABLE 12

Comparative Uptake of Nucleic Acid Precursors by L929 and its 2 Sublines L929SE$^-$† and LA9*

Substrate	Cell Line		
	L929	L929SE$^-$	A9
	Transport in pmol/min/10^6 cells		
Adenine	25	100	0
Hypoxanthine	15	50	0
Uracil	0	0	0
Cytosine	0	0	0
Adenosine##	75	160	68
Inosine	12	40	6
Uridine	3	105	1.5
Guanosine	10	not tested	2

† grown in serum-free medium

*selected for H PRT'ase$^-$ but also found to be A PRT'ase$^-$

##U-^{14}C-adenosine used.

(The data in this table were kindly contributed by Dr. J. Li of this laboratory.)

TABLE 13

Uptake of Nucleic Acid Precursors by BHK/21 Cells and Membrane Vesicles (pmol/min/10^6 Cells or Derivative of 10^6 Cells)

Substrate	Intact Cells	PM + ER Vesicles
Adenine	112	0.24
Adenosine	88	0.45
Hypoxanthine	100	1.03
Uracil	65	1.62
Uridine	2.4	0.82

TABLE 14

Concentration Against A Gradient of Metabolites Transported by Isolated BHK Membrane Vesicles

Substrate	Concentration factor observed*
Glucose	4.0
Adenosine	58.0
Adenine	18.2
Hypoxanthine	14.5
Uridine	25.0
Uracil	6.3

*Performed at single substrate concentration, thus represents minimum concentrating abilities of the systems involved.

cell rate than we observed in bacterial systems (cf. References 4, 44). Exposure of new transport sites on internal membranes could account for this.

In order to determine whether a facilitated diffusion process or a true active transport (defined as concentration against a gradient) was operative we measured the concentrating power of the vesicles. This was done by determining extravesicular versus intravesicular space in the reaction mixture (inulin vs. urea space) as had been done previously in the bacterial systems.[4] This internal space was found to be 1.88 μl/mg protein for crude microsomes, so that in washed microsomal vesicles the internal space would be approximately 5 μl/mg protein, similar to the ratio for washed bacterial vesicles.[4] The concentration factors seen in Table 14 are minimum estimates since the factors were calculated from experimental data gathered during linear initial rates of transport. Maximum concentrating power can be determined at low substrate concentration (Table 15) after long intervals at 25°C, as was done for adenosine uptake.

Though the phosphoribosyltransferases are important to the regulation of purine transport in animal cells (Lesch-Nyhan disease is detected in the fetus by failure of amniotic cells to take up hypoxanthine,[87] it at first appeared that the mechanism of adenine uptake differed from that observed in bacteria,[4] based on the distribution of intravesicular products identified by thin layer chromatography: unreacted adenine 54%, adenosine 19%, and AMP 27%. This distribution instead may reflect high activity of degradative enzymes associated with the vesicles (i.e. 5′ nucleotidase). It is currently believed that the PRT'ases are involved in uptake, and that these results are due to 5′ nucleotidase activity. This was deduced from uptake patterns for all purine bases and nucleosides in purine PRT'ase deficient lines, as will be discussed subsequently. All enzymes of initial nucleic acid precursor metabolism were determined for the vesicles so as to limit or widen possible utilization routes to be considered; the results of these determinations are summarized in Table 16.

Adenine uptake in vesicles from BHK cells is approximately 12 pmol/min/mg vesicle protein for crude microsomal vesicles (Figure 20). The concentration dependence of oligomycin inhibition of adenine uptake is similar to that for other nucleic acid precursors (Figure 21), and so

TABLE 15

Adenosine Uptake Into BHK Vesicles

K_m Observed 70 μM ± 20 μM (4 Determinations)
V_{max} Calculated 91.5 pmol/min/mg Vesicle Protein

Initial Rates of Adenosine, Concentrations Employed

Concentration	Initial velocity pMol/min/mg vesicle protein	Maximum concentrative power of vesicles
2 μM	5.0	58-Fold
50 μM	33	5.6-Fold
100 μM	47.8	4.3-Fold
2 mM	235	None

TABLE 16

BHK Membrane Vesicles: Enzyme Activities

Enzymes present on vesicles	
Ad: PRT'ase	Ad + PRPP → AMP + PP_i
Adenosine deaminase	Ads → Inos + NH_3
Adenosine phosphorylase	Ad + Ribose -1- P ⇌ Ads + P_i
Inosine phosphorylase	Hx + Ribose -1- P ⇌ Inos + P_i
Uridine kinase	Urid + ATP → UMP + ADP
Enzymes absent on vesicles	
Adenosine kinase	Adenosine + ATP → AMP + ADP
Uracil PRT'ase	Uracil + PRPP → UMP + PP_i
Uridine phosphorylase	Uracil + RIP ⇌ Uridine + P_i
Adenylosuccinic synthetase	
Adenylosuccinase	IMP → AMP
Enzymes present in trace amounts	
Hx PRT'ase	Hx + PRPP → IMP + PP_i
Inosine kinase	Inos + ATP → IMP + P_iDP

suggests an effect on membrane integrity or common energy coupling rather than on the adenine transport system itself.† (Oligomycin is known to inhibit the ouabain sensitive, Mg^{2+} dependent, Na^+ K^+ stimulated plasma membrane ATP'ase.) This is in contrast to the finding of differential inhibition by N- ethylmaleimide or p-chloromercuribenzoate on adenine, uridine and uracil uptake (Figures 22 and 23). These results indicate that individual transport carriers are involved, and that the sulfhydral compounds have specific effects on each, while the oligomycin interferes with a separate site common to the three transport systems. Further investigation of effectors of adenine transport in the vesicles indicated significant inhibition by other uncoupling agents, electron-transport inhibitors, and chelating agents (cf. Figure 24). Such inhibitors are useful in developing, defining, and characterizing the systems, but have no bearing on in vivo control mechanisms, so that possible feedback effects of pathway end products were also examined (Figure 25). The low level stimulation of adenine uptake by GTP with inhibition at higher concentrations has been observed by us in a number of other adenine uptake systems[3,8] and the mechanisms possibly accounting for such an observation have been discussed elsewhere.[3,8] ATP inhibits at all levels, with no such stimulatory effect.

†These vesicles prepared by the Wallach technique[79] are virtually free of mitochondrial contamination and the oligomycin effect appears to be from its action on surface or endoplasmic membranes in this instance.

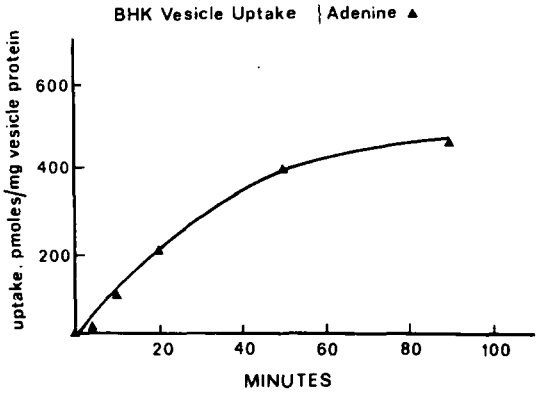

FIGURE 20. Vesicle uptake of adenine by baby hamster kidney C13 cells. Reaction mixtures: 250 μl, containing 1 mg purified membrane vesicle protein, sucrose (.05 *M* final), potassium phosphate (.05 *M*, pH 7.5), $MgSO_4$, (1 m*M*), and U-^{14}C-adenine (231 mCi/mmol: Amersham Searle) at final concentration of 50 μM were incubated at 35°C for the times indicated and the reactions were terminated by dilution to 2 ml with 0.8 *M* NaCl at 35°C and rapid filtration on 0.3 μmpore-size filters (Millipore). Filters were dried and counted as described.[4,5]

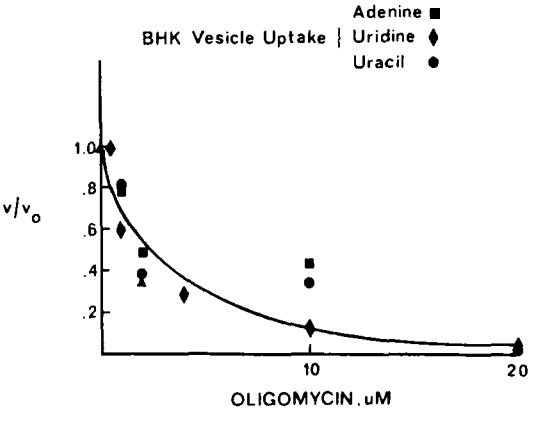

FIGURE 21. Effect of oligomycin on membrane vesicle uptake in isolated membrane vesicles of baby hamster kidney cells. Substrates used were 2-^{14}C-uridine specific activity 50 μCi/μmole (Amersham Searle) 2-^{14}C-uracil specific activity 52 μCi/μmole (Amersham Searle) and 8-^{14}C-adenine 50 μCi/μmol (Amersham Searle). Each substrate was used at 0.1 m*M*, the initial uptake rates for the sample which received no oligomycin were 16 pmol/min/mg for adenine, 27 pmol/min/mg for uridine, and 11 pmol/min/mg vesicles for uracil. Incubation time was 15 min during which linear rates of uptake were observed. The results are represented as fractional inhibition in the presence of 0.5, 1, 2.5, 5, 10, and 20 μ*M* oligomycin concentrations. Other details are as for Figure 20.

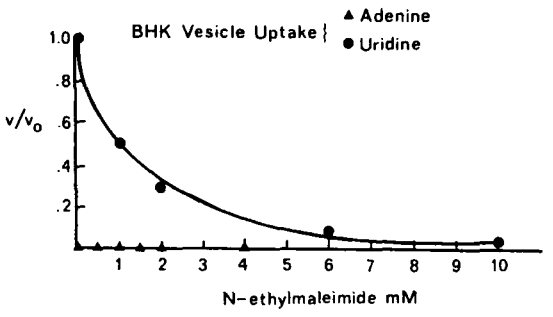

FIGURE 22. Effect of N-ethylmaleimide on adenine and uridine uptake in baby hamster kidney C13 membrane vesicles. Details of the experimental condition are similar to those described in Figures 20 and 21.

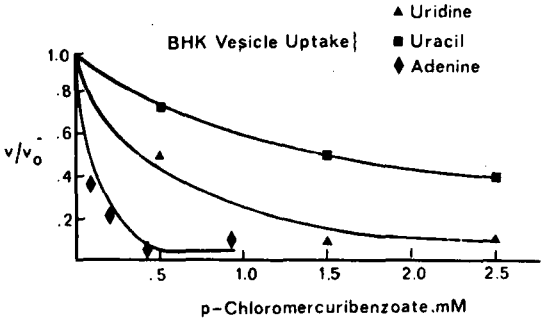

FIGURE 23. Effect of p-chloromercuribenzoate (pCMB) on vesicle uptake of nucleic acid precursors in membranes purified from BHK cells. See Figures 20 and 21 for experimental details.

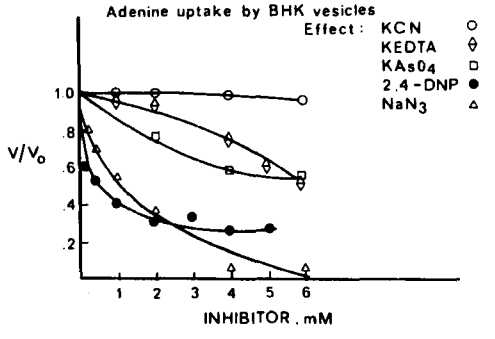

FIGURE 24. Effect of metabolic inhibitors on adenine uptake in BHK vesicles. See Figures 20 and 21 for experimental details.

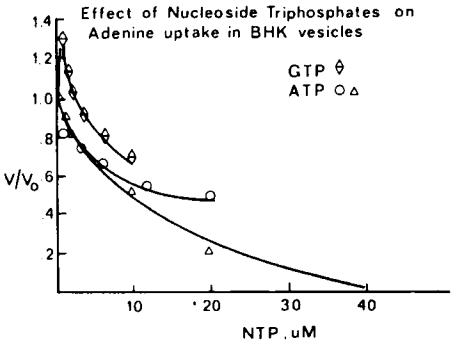

FIGURE 25. Effect of nucleoside triphosphates on adenine uptake in BHK vesicles. Experimental details the same as in Figures 20 and 21. Two determinations measuring effects of ATP are shown to indicate level of reproducibility of assay.

The results presented thus far establish the properties of a vesicle transport system for membranes isolated from mammalian cells in culture and begins to shed new light on uptake mechanisms operative in animal cells.

It seems that isolated membrane vesicles are capable of transport. Thus the original finding of Wallach and co-workers[86a] that membranes isolated by the cavitation technique are continuous and osmotically intact are confirmed by our measurements of internal space and by their ability to concentrate certain substrates. We cannot yet define the concentrative uptake observed as being due to classical active transport or group translocation, for it could result from facilitated diffusion coupled to an enzymatic conversion in a way that requires energy. Though the first enzymes of the metabolic pathway are well represented on the isolated membrane vesicles, they may function subsequent to uptake because unmodified substrate is observed intravesicularly after very short reaction times. Moreover, it is difficult to differentiate products of 5′-nucleotidase action from uptake of free base.

b. Hypoxanthine Utilization

The mechanism of hypoxanthine utilization would seem to be most easily elucidated, due to the great availability of appropriate mutant lines. Hypoxanthine-guanine PRT'ase (HG PRT'ase) is one of the two best selection systems available for mammalian cell lines, the other being thymidine kinase.[99,100] Mutants lacking the enzyme are easily recovered after growth on 8-azaguanine or 6-thioguanine. Revertants can be isolated in HAT (hypoxanthine-amethopterin-thymidine) medium[101] on which cells lacking a functional hypoxanthine utilizing system do not grow.[102,103] Some of the mutants include human diploid fibroblast lines from patients with Lesch-Nyhan disease, a sex-linked genetic defect identified as being caused by missense mutations in the structural gene for HG PRT'ase.[87,105] Numerous reviews have dealt with the HG PRT'ases and the reader is referred to these papers for details of the extensive studies of the enzyme.[106-108] A few summarizing remarks necessary to our discussion are appropriate:

1. HG PRT'ase isozymes exist, in that isoelectric focusing can be used to isolate several forms in normal cells.[16]
2. Total activity is lacking in mutant cells, indicating that a common subunit shared by isozymic forms or that polymerization of identical monomers is responsible for the isozyme pattern observed.[104]
3. Cells selected in 8-azaguanine may lack HG PRT'ase or may still possess the enzyme. Hypoxanthine transport under both circumstances is absent.[102,103]

Several stable variant lines have been isolated which retain HG PRT'ase activity in extracts, but no longer transfer hypoxanthine label into nucleic acids.[102,103] This can be explained if hypoxanthine transport and the HG PRT'ase are distinct, with these mutants are defective only in the transport system. A similar line of reasoning was used when the same circumstances were first encountered with bacterial systems.[17] However, in the bacterial system a direct dependence on each of two HG PRT'ases has demonstrated one isozyme is necessary for hypoxanthine uptake and another for guanine uptake as already discussed.[10-12]

Certain preliminary observations suggest that the mechanisms of hypoxanthine uptake in mammalian cells and enteric bacteria are quite similar. PRPP stimulates hypoxanthine uptake tenfold in vesicles prepared from Balb/c 3T3 cells,[110] but no uptake is measurable in vesicles from a subline selected to be resistant to 6-thioguanine (Quinlan and Jha, to be published) nor in A_9 cells (HG PRT'ase$^-$), whether as the free base or from the nucleoside.[78] Reversion to HG

PRT'ase$^+$ in the A_9 line[88] restores hypoxanthine and inosine transport in vesicles with accumulation of inosine and some IMP. The elevated intravesicular concentration of nucleoside may be attributable to IMP hydrolysis by plasma membrane 5′ nucleotidases.

It is difficult to postulate the participation of membrane HG PRT'ase in a group translocation if hypoxanthine is purportedly still utilized in some mutants apparently lacking HG PRT'ase activity. The criteria for defining this group of mutants, however, are 8-azaguanine resistance, incorporation of hypoxanthine into cellular nucleic acids, and absence of HG PRT'ase activity in cell extracts prepared by freeze-thaw. Because incorporation rather than uptake is usually measured, it has been postulated that though the enzyme is functional *in situ* (in order for nucleotide formation to occur), it is not functional in the cell extracts.[103] Since the specificity of the selection system precludes an alternative pathway,[99-105] the conclusion of Beaudet et al.[103] that the enzyme must function *in situ* seems plausible. These workers must have selected mutant enzymes which differ from the wild-type in resistance to freeze-thaw and in ability to complex 8-azaguanine. An analogous situation has been observed for the *Salmonella* enzymes in that selection of 8-azaguanine resistant mutants leads to recovery of mutants with HG PRT'ase having lower affinity for the analogue and different physical properties than the wild-type (new chromatographic behavior).[28,109]

Correlation of guanine and hypoxanthine uptake, and their respective uptake systems is always limited by purely technical considerations. Perhaps as a result of its lack of water solubility, guanine usually sticks to filters often used for uptake assays, and thus makes specific uptake difficult to measure, even when filters are prewashed with the unlabeled base.[10-12] Thus, data are usually obtained for hypoxanthine and, less so, for guanine *conversion* by HG PRT'ases, but *uptake* is usually almost exclusively assayed for hypoxanthine. This situation is once again exemplified in a recent study which measures transport of hypoxanthine alone into fibroblasts with normal and mutant hypoxanthine-guanine phosphoribosyltransferase.[109a] Though all study of guanine uptake is omitted, results obtained show a very clear and precise correlation between hypoxanthine uptake and enzyme presence in normal and mutant cells. Normal cells grown on amethopterin were increased in hypoxanthine uptake rates. In bacteria, growth in amethopterin results in increased hypoxanthine phosphoribosyltransferase activity (Tables 2 and 3). Though the present understanding of hypoxanthine uptake by mammalian cells is still incomplete as to precise mechanism (i.e., does PRT' ase act in the transport step or in an immediately following step?), the relationship of PRT'ase activity to uptake should soon be elucidated through the use of isolated mammalian membrane vesicle transport systems[77,78,110] and the full complement of mutants and revertants available.[88,102,103,109a, and Quinlan and Jha, to be published]

2. *Purine Nucleoside Uptake*

Conclusions concerning the uptake of purine nucleosides into animals – *Though it is not clear whether cleavage of purine nucleosides is obligatory or even the predominant first step in their utilization in animal cells, the use of isolated vesicles from a variety of established cell lines (for study of transport in this laboratory) has indicated that cleavage occurs to a significant extent. At low substrate concentrations exposure of vesicles to nucleoside usually resulting in a greater accumulation of intravesicular ribose-1-P than of any other compound. In whole cells the transport of purine nucleosides cannot be dissociated from their appearance in the cytoplasmic pool as monophosphates. The experiments leading to these conclusions are detailed below.*

a. Adenosine Utilization

There have been a considerable number of studies investigating the properties and regulation of adenosine uptake in a variety of animal cells.[66,68,77,78,89-96] Some of these reports do not attempt to distinguish uptake of the sugar and purine moieties when using uniformly labeled adenosine,[90,93] though this was done in one careful study.[66] In some cases the products of uptake were ill-defined,[90] but most workers chose to analyze uptake products extensively.[66,77,78,91,94-96] Certain generalizations are apparent from these results: There seems to be considerable heterogeneity among cell types and lines as to whether adenosine is deaminated as the first step in its utilization.[66,78,89,92,94] Growth conditions and the status of other pathways in the cell undoubtedly affect deaminase function. In this laboratory, we have found (Li and Hochstadt, to

be published) that membranes from A_9 cells lacking adenine PRT'ase (A PRT'ase), hypoxanthine PRT'ase, and unable to utilize any exogenous purine moiety from either base or nucleosides, also have much lower adenosine deaminase activity. A revertant of this line with restored HG PRT'ase also partially regains membrane adenosine deaminase activity, while the original parent line of both, 929, has sufficient deaminase activity to rapidly convert virtually all adenosine to inosine prior to uptake. Thus, in 929 it has been most difficult to study an adenosine transport system per se if it exists.[78] Thus, when plasma membrane vesicles of 929 are exposed to adenosine, IMP and ribose-1-P, and small amounts of inosine accumulate within the vesicles. Large amounts of inosine and hypoxanthine appear in the medium and no AMP or adenosine is observed in the vesicles. Detailed presentation of the kinetics and products of nucleoside uptake in 929, 929^{se-} (subline which grows on completely defined media), A_9 (929 derivative lacking all purine PRT'ase activity), and $A_9{}^R$ (revertant for hypoxanthine PRT'ase) will be published elsewhere (Li and Hochstadt, to be published).

Two of the above cited investigations are of particular further interest. Taube and Berlin[66] examined adenosine uptake by rabbit polymorphonuclear (PMN) leucocytes. The conclusion that the initial step in adenosine utilization by these cells is a deamination to inosine followed by hydrolysis to hypoxanthine, was obtained from the results of a series of double isotope experiments which traced the separate fate of the purine and ribose moieties of the nucleoside. Cells were exposed to ^{3}H-adenosine labeled in the base and uniformly labeled ^{14}C adenosine cells in 10:1 ratio of dpm (disintegrations per min) (10:1 dpm ^{3}H:^{14}C). The ratio of ^{3}H:^{14}C dpm associated with the cells was then determined after uptake. If adenosine is taken up intact, then the ratio of 10:1 would persist. Since no cellular adenosine phosphorylase activity was detectable while inosine phosphorylase was abundant, they argued that alterations in the ratio of ^{3}H:^{14}C would result from cleavage of inosine to hypoxanthine and ribose-1-P. If the ratio of ^{3}H:^{14}C dpm were to increase, more of the adenosine purine moiety would be used than the ribose moiety, and some cleavage would have had to occur. The ^{3}H:^{14}C ratio observed was 25:1, even somewhat greater than would be expected (20:1) if all adenosine were first cleaved into free purine bases and sugar (or sugar phosphate), and then only the base were utilized for nucleotide formation.

Taube and Berlin further postulate that, at least in the rabbit PMN leucocytes, adenine and adenosine transport systems are separate because adenine is only a poor inhibitor of adenosine uptake. This conclusion is unwarranted, since the results of that paper clearly indicate via the elegant double isotope experiment that adenosine is being taken up exclusively as hypoxanthine. Since they also found adenosine phosphorylase lacking, adenine cannot enter this pathway via conversion first to adenosine. Thus, what Taube and Berlin have demonstrated is that adenine and hypoxanthine have distinct transport systems.

Experiments from the author's laboratory have shown that cell lines both lacking adenine and hypoxanthine PRT'ase activity are incapable of adenine transport, whether as the free base or from the nucleoside.[78] A revertant line (for only the hypoxanthine PRT'ase, but not the adenine PRT'ase) is restored to the ability to use base from either inosine or adenosine which is deaminated to inosine (Li and Hochstadt, to be published). Thus, the caveat should be reiterated that the relationship of adenine and adenosine uptake in any cell line might be completely determined by levels of adenosine phosphorylase and adenosine deaminase.

Such ambiguities are likely to occur whenever possible "pre-uptake metabolism" of a substrate goes unaccounted for which further emphasizes the possibility of heterogeneity in uptake mechanisms for different mammalian cell types, and underscores the necessity of employing specifically labeled precursors and isolating and identifying both uptake products and untransported radioactivity. The extent and variety of initial pathway reactions that occur in whole cells is somewhat more restricted when vesicle uptake is studied, but even with vesicles the variety of products potentially recoverable requires careful analysis of both vesicle contents and materials in the extravesicular medium.

Taube and Berlin also compared the inhibitory properties of cytidine and the unnatural nucleoside isoguanosine on adenosine transport. The two are structurally very similar, having identical sugar and pyrimidine moieties but differing in that isoguanosine is a purine nucleoside with the glycosidic bond at N-9. Consequently, the ribose is

displaced ~ 2.5 Å in isoguanine relative to cytidine. Despite this configurational difference, the two compounds are near equivalent as inhibitors of adenosine uptake. Taube and Berlin propose a flexible transport carrier that would accommodate this 2.5 Å displacement and so recognize the structural similarities in the molecules. A simpler explanation might be that the bases alone are inhibitory once the glycosidic linkages are cleaved so that the 2.5 Å displacement vanishes. These results further emphasize the necessity to determine whether effectors are recoverable intact or have themselves been metabolized to other compound(s) which are in fact responsible for the regulation noted. Thus, though the study of Berlin and Taube indicates a pathway of adenosine utilization, their contention that the pathway initially involves uptake of intact nucleoside by a carrier common for adenosine and thymidine seems yet to be unequivocally demonstrated for rabbit PMN cells (in which the proposed mechanism might indeed be the correct one), as well as for other cell types.

Plagemann[95] showed that it is possible to greatly expand the nucleotide pools in Novikoff rat hepatoma cells. The adenosine is largely recoverable in the nucleotide pool as adenine nucleotides. At the high external concentrations employed (1 m*M*), passive diffusion is likely to play a significant role,[77] so it is likely that phosphorylation occurs subsequent to permeation through the membrane under these conditions. These cells must be capable of phosphorylating free intracellular base or nucleoside because of the finding of a *large* pool of nucleotides rather than unphosphorylated compounds intracellularly. These nucleotides are not used for macromolecular biosynthesis, however, despite the size of the pool, since subsequent exposure to labeled adenosine caused low levels of the exogenous nucleoside to be rapidly incorporated into newly formed nucleic acids. The large intracellular pools formed during exposure to high exogenous adenosine concentrations can be depleted when the cells are energy starved.[94] When the cells are exposed to glucosamine,[96] the glucosamine is rapidly taken up, phosphorylated, and the cellular ATP pool is largely converted to ADP and AMP. It is not understood why the accumulated cellular ATP pool is not used for nucleic acid synthesis, while newly transported adenosine is exclusively shunted to macromolecular synthesis. The mechanism which precludes admixture of newly taken up adenosine with cytoplasmic nucleotides is also unknown. A rather simple model to explain these phenomena is the postulation that the nuclear membrane excludes ATP, but some metabolite earlier in the pathway from adenosine to ATP (e.g., AMP) is the compound which permeates to the nucleus. Thus, AMP arising from either new adenosine or the eventual breakdown of cytoplasmic ATP and not directly arising from the cytoplasmic ATP pool would gain access to nuclear processes.

Plagemann and Erbe studied adenosine transport in KCN-treated cells.[94] When normal cells take up either uridine or adenosine, the substrates are rapidly recovered as UTP and ATP, and only small amounts of UDP, UMP, ADP, or AMP and no free bases or nucleosides are usually detected. In KCN-treated cells, however, uridine uptake is considerably diminished, no concentration against the gradient can be observed, and the nucleoside is recoverable from the cell unchanged. Adenosine, however, continues to be taken up at high rate, is concentrated against the gradient, and is recoverable as AMP (with some ADP). Thus, KCN prevents conversion of adenosine taken to ATP, though AMP formation continues. Because metabolic inhibitors are incapable of inhibiting nucleotide formation from bases in the presence of sufficient PRPP,[5] the observation that in KCN-treated cells, adenosine is concentratively taken up and recoverable as AMP while uridine is only equilibrated and recovered unchanged would indicate separate mechanisms. In the case of adenosine it is likely that the A PRT'ase is involved[78] and that the PRPP requirement can be met in the absence of metabolic energy due to PRPP stores that are membrane localized. (Figure 24 shows that adenine uptake in baby hamster kidney cell vesicles is also not inhibited by KCN, as is also observed in enteric bacteria.[4,5]) With uridine as substrate the situation seems quite different, however; few animal cell lines (BHK is a notable exception,[97]) can cleave uridine to uracil and use the base. The uridine is undoubtedly taken up intact in most animal cells as will be discussed later, and would explain the differential inhibition by KCN.

Adenosine and adenine uptake differ, however, in mouse Balb/c 3T3 cells (Quinlan and Hochstadt, submitted for publication). While adenosine uptake seems uninfluenced by confluence of the culture, a

result consistent with that of Cunningham and Pardee,[98] adenine (and uridine) utilization is considerably diminished when the cell growth is contact-inhibited. Moreover, in Balb/c 3T3 and Balb/c SV40 3T3 vesicles, uptake of free base (e.g., hypoxanthine[110]) is dependent upon added PRPP while nucleoside transport is not (Quinlan and Hochstadt, to be published). This observation is reminiscent of bacterial purine nucleoside transport in which the ribose moiety appears conserved for preferential utilization in intracellular formation of nucleoside.[25]

b. Inosine and Guanosine Utilization

An active inosine uptake system in Novikoff cells appears operative in that labeled inosine can be taken up into the cellular nucleotide pool at seven times the rate of incorporation of label into nucleic acids.[95] There appear to be two routes of inosine utilization in such cells. When RNA is hydrolyzed to detect the fate of inosine label, ~82% is found as GMP, but in the acid soluble nucleotide pool, most of the label is recovered as ATP. The route of processing thus seems to be determined by enzyme activities localized at different sites within the cell. In the cytoplasm, inosine is apparently restricted to enter the adenine nucleotide pool while inosine that reaches pathways associated with the nucleus is shunted to GMP. Either inosine itself, if taken up unchanged, or IMP must be the compound that is transported to the nucleus, though transport systems for endoplasmic reticulum caniculi and/or the nuclear membrane are yet to be demonstrated. There is also considerable difficulty in distinguishing an inosine uptake system from the processing of deaminated adenosine, and in discerning whether inosine is broken down to hypoxanthine prior to uptake.

Plagemann's studies reveal that though there is substantial conversion of adenosine to inosine and then more slowly to hypoxanthine, adenosine itself is utilized by Novikoff cells, since fewer cellular guanylate residues of RNA contain label from adenosine tracer than from inosine tracer.[95] Uptake of adenosine is markedly less sensitive to inhibition by guanosine than is the inosine uptake. Though uptake of adenosine is more sensitive to competitive inhibition by phenethyl alcohol than inosine uptake, this could be used to distinguish the two systems only if it were shown that adenosine deaminase is *not* sensitive to the effector. It is entirely possible, however, that guanosine and inosine are taken up by a single system, and this is supported by inhibition patterns observed with several cell lines.[90-92]

In my own laboratory we have consistently observed a most interesting situation in L 929 cell membrane vesicles, A_9 cell membrane vesicles (HG and A PRT'ase$^-$), and A_9^R (A_9 revertant only for the HG PRT'ase) in the uptake of inosine as compared to adenosine. As mentioned previously, the A_9 membrane vesicles take up predominantly only the ribose moiety of uniformly labeled adenosine or inosine though some free inosine is observed intravesicularly.[78] The surrounding medium contains adenine and hypoxanthine, and hypoxanthine, respectively, though the finding that adenine results from exogenous adenosine differs from the data obtained by Taube and Berlin[66] with PMN cells. The A_9 mouse line contains only 3 to 6% of the adenosine deaminase activity expressed by a parent 929 "L" cell line. Thus, the discrepancy may reflect the absence of adenosine phosphorylase for other cells and cell lines, or a difference in adenosine deaminase activity. The fact that the specific activity of adenosine deaminase is high is exemplified by our own observation that in an A_9 revertant for HG PRT'ase (A_9^R), which expresses only 16% of the wild-type deaminase activity, no free adenine can be observed in the surrounding medium.

Perhaps the most paradoxical of our findings[78] is that with membranes having associated deaminase activity, and also retaining A PRT'ase and HG PRT'ase (i.e., wild-type 929), adenosine is the preferred substrate though inosine (and not adenosine) is always found inside the vesicles. This may be analogous to the substrate processing previously detected for nucleosides[6] or may simply reflect two independent uptake systems for adenosine and inosine with rapid deamination subsequent to uptake. Vesicles prepared from the wild-type certainly contain a level of deaminase activity to preclude measurement of intravesicular adenosine. This latter hypothesis is unlikely because in whole cells deaminated product appears rapidly in the medium (Li and Hochstadt, unpublished data). Furthermore, PRPP does not seem to significantly stimulate uptake of either purine base or nucleoside in "L" cell vesicles, and in this respect, they differ from the Balb/c 3T3 vesicles.

Though group translocation is less tenable in L cells than in Balb/c 3T3, however, the most significant evidence to date on the direct involvement of phosphoribosylation in purine uptake in L cells comes from work with mouse fibroblast vesicles from L_{929} and A_9 sublines performed in this laboratory by Dr. J. Li.[78] The A_9 subline of L cells is an A PRT'ase negative, HG PRT'ase negative line. It is unable to take up adenine or hypoxanthine. When vesicle transport of uniformly labeled adenosine or inosine is studied, however, the products recovered in the vesicles are ribose-1-PO_4 and some inosine. Nevertheless, the parent L cell line, 929, accumulates IMP, ribose-1-P and again small amounts of free inosine. Failure to recover adenosine or AMP when adenosine in the substrate is attributed to the very active adenosine deaminase found associated with the outer surface of the vesicles. Thus, it would seem that these results suggest that either the hypoxanthine phosphoribosyltransferase is involved in membrane transport of precursors, or that the enzyme and transport functions are both genetically controlled by the locus reverting to regain hypoxanthine PRT'ase activity, whereby it would be reasonable for transport and enzyme functions to be lost and regained together, but that this locus is not necessarily the structural gene for HG PRT'ase in A_9. It seems essential at this time, therefore, to analyze additional mutants and revertants to test this very seemingly remote, but still *much* more probable possibility (than multiple mutations and reversions). Luckily, this system offers more available mutants and revertants for a variety of cell types than any other biochemical marker, whatsoever, in mammalian cell lines in culture, and in a number of the cell lines the defect is known to reside in the structural gene for HG PRT'ase.[103,104]

Nucleoside transport into human erythrocytes is reported to occur by facilitated diffusion and is inhibitable by *p*-nitrobenzylthioguanosine, but uptake of the free base is unaffected by such compounds. The inhibitors may act on the nucleoside phosphorylases. Though a stabilizing effect of purine nucleosides on human erythrocytes has been noted,[112] this may reflect the value of the ribose moiety to the red cell rather than represent a preference for the intact nucleoside over the free base.[112,112a]

B. Pyrimidines

1. Pyrimidine Base Uptake

Conclusions concerning utilization of pyrimidine basis in animal cells – *Free pyrimidine bases are not well utilized by animal cells. There is suspicion when they are taken up at all that this is evidence of mycoplasma contamination. Exceptions to this, however, are certain cell lines derived from kidney tissue. Here again either the kidney tissue is more subject to contamination in vivo or kidney cells normally express enzyme activities not generally observed in other tissues. The utilization of uracil presumably requires the presence of uridine phosphorylase. The present state of knowledge in this field is outlined below.*

It is commonly believed that pyrimidine bases are not utilized in animal cells. Some confusion has occurred because marked increases in utilization, around the periphery of cells in particular, has been noted in cultures contaminated with mycoplasma.[113] Uracil and orotate uptake by ascites cells has, however, been detected by in vitro assay with primary cultures[114] or fresh explants,[115] so that contamination by mycoplasma is less likely than in established cell lines. In these studies it was found that uracil and orotate may be taken up by separate processes. Cells respond to increases in glucose by taking up more uracil, but without similar elevation of orotate uptake. This differential stimulation by glucose could, however, occur were orotate transported via orotate PRT'ase but uracil by uridine phosphorylase and uridine kinase.

With freshly explanted tumor cells, a most interesting observation has been made concerning the regulation of orotate uptake.[115] Incorporation in whole cells could be stimulated more than 90-fold by a combination of PRPP and a heat labile, and nondialyzable material whose activity in extracts parallels orotate PRT'ase activity. Except for the possibility that orotidine monophosphate itself is the preferred substrate for transport, these observations suggest that exogenously added orotate PRT'ase might attach to the membrane and in the presence of external orotate and PRPP result in the formation of internal OMP. If such a mechanism were operative in ascites cells, it would be analogous to the mode of adenine uptake in enteric bacteria.[4,5]

Uptake of uracil by several cell lines has been assayed in my laboratory (Tables 11 and 12). "L" cells, Chinese hamster ovary (CHO) cells, and

HeLa cells do not transport uracil, but uptake was noted in a 3T3 culture and, to a much greater extent, with the BHK C13 line. The data reported in Table 11 for a Swiss 3T3 cell suspension have not been validated for freedom from mycoplasma infection, though all our subsequent work on Balb/c 3T3 cells has shown no contamination by culture assay. The BHK line is also regularly studied in this laboratory and regularly tested for mycoplasma infection.

BHK C13 cells have a high uridine phosphorylase activity.[97] In isolated vesicles, uracil uptake is only about 2% of the uptake seen in whole cells (Table 13), undoubtedly due to coupled processes or further utilization. Destruction of uptake sites during preparation may also be significant factors as great variability in uracil uptake was observed among batches with many preparations lacking uracil uptake activity. Uracil is concentrated against the gradient (Table 14), though to a lesser extent than any other nucleic acid precursor and, in fact, only slightly more so than glucose which has been reported not to be concentrated in membrane vesicles from rat fat cells. (However, the cold, low ionic strength washes employed in the technique used for these reports might lead to material "leaking" from the vesicles even if concentrated.[86]) Uracil uptake is inhibited by oligomycin to the same extent as uridine and adenine uptake, suggesting a possible common energy coupling or energy requirement for membrane integrity (Figure 21). Inhibition of uracil uptake by pCMB is minimal in comparison with the effects on adenine or uridine uptake, suggesting a separate uptake mechanism (or carrier).

BHK cells would seem anomalous among mammalian cell lines in their ability to use uracil. A rat kidney line and other kidney lines now also seem to possess uridine phosphorylase activity. The only kidney line lacking uridine phosphorylase is B-1, selected for its resistance to bromodeoxyuridine.[116] The possibility does exist, however, that the kidney is more vulnerable to mycoplasma infections than other tissues so that *in situ* mycoplasma infections could be responsible for uridine phosphorylase in mammalian cells. The ubiquity of mycoplasma infections in cell lines, occasionally subtle and difficult to detect, may pose a serious limitation to transport studies. Such problems seem best solved at this time by continued autoradiographic monitoring to ascertain that uracil taken up is incorporated predominantly in the nucleus and not at all around the cell periphery. In addition, careful electron microscopy of cell fractions and sections should provide definitive information about uracil transport in kidney and other cells, and membrane vesicles derived therefrom.

Thymine uptake as a free base has not been described as a mediated process in animal cells. Thus, uptake of free thymine might prove particularly useful as an assay of mycoplasma contamination.

2. Pyrimidine Nucleoside Uptake

Conclusions concerning the uptake of pyrimidine nucleosides into mammalian cells. – *Pyrimidine nucleosides can be taken up intact and in all probability this is their only mode of uptake. Under certain experimental conditions (KCN treated cells) uridine uptake can be dissociated from its phosphorylation indicating that UMP formation occurs subsequent to, rather than in the process of, transport. Thymidine phosphorylation has not yet been dissociated from its mediated transport so it is not yet known whether thymidine kinase, the enzyme necessary for thymidine utilization, acts during or subsequent to transport. Cytidine seems not to be utilized unchanged. The experimental results upon which these conclusions are based are detailed below.*

a. Uridine Utilization

Plagemann has contributed significantly to the understanding of uridine utilization in animal cells, particularly in Novikoff hepatoma cells.[91,93,94,118] Uptake has been dissociated from phosphorylation by the use of KCN-treated cells,[91] which, as previously noted, accumulate free uridine while adenosine is concentrated as AMP. Plagemann was also able to determine what effect precursor incorporation into nucleic acids has on the rate of uptake itself by taking advantage of the observation that uptake continues at ~5°C while RNA synthesis ceases under such conditions. With external uridine concentrations of 0.5 m*M*, UTP could accumulate within the cytoplasm to levels of 5 m*M* concentration when RNA synthesis was terminated. Persantin and adenosine were observed to inhibit incorporation of uridine into macromolecules, but did not inhibit phosphorylation by uridine kinase in extracts.[118] Since K_m for uridine uptake is an

order of magnitude lower than K_m for uridine kinase, uptake was judged a separate function from the kinase and the rate-limiting step in uridine utilization.

After accumulation of the cold-expanded UTP pool and shift-up in temperature, exogenously administered uridine with a different isotopic label was observed to be exclusively incorporated into newly synthesized RNA. From this, Plagemann deduced the existence of two distinct immiscible nucleotide pools, one in the cytoplasm and one in the nucleus, with only the nuclear pool in equilibrium with the outside of the cell. It was not suggested, however, how this might occur. The caniculi of endoplasmic reticulum, which traverse the cytoplasm from nucleus to cell membrane, may constitute the structural basis for this pool separation. Alternatively, if the nuclear membrane permits only selective entrance of pool components, then a separation of nucleotide pools could occur without a particular structural requirement. If the nuclear membrane was permeable to any or all of the uridine precursors except UTP, and if the cytoplasmic energy relationships were such that uridine precursors remaining in the cytoplasm were rapidly converted to UTP (as is the case) then it can be understood how exogenous uridine would preferentially appear in RNA despite the presence of a large cytoplasmic UTP pool. Since UTP itself does not permeate the nucleus in such a scheme, the cytoplasmic UTP pool would only be used slowly after being converted to UMP or UDP.

The mechanism of uridine uptake is likely to vary considerably among cell lines and with growth conditions. Cunningham and Pardee[119] have observed a 20-fold difference in uridine uptake between contact-inhibited 3T3 cells and the same cells treated with fresh serum for 5 min under conditions which initiate the reversal of contact inhibition and will result in a round of subsequent DNA synthesis. In this laboratory we have been able to confirm that this difference in uridine uptake between growing and nongrowing cells is controlled at the level of the membrane itself. Purified plasma membrane vesicles prepared from subconfluent cultures of Balb/c 3T3 transport uridine at several-fold higher rates than vesicles prepared from confluent cultures. Adenosine transport rates by the two vesicle preparations are quite similar, again similar to the observations of Cunningham and Pardee[119] (and Quinlan and Hochstadt, submitted for publication).

Uridine uptake is also observed to be more extensive in 3T3 cells transformed with SV40, an oncogenic virus,[120] than in the normal contact-inhibited counterparts. In my own laboratory, we have also observed this difference in the related cell lines, Balb/c 3T3 and SV40 Balb/c 3T3 (Quinlan and Hochstadt, submitted for publication). Moreover, SV40 Balb/c 3T3 plasma-membrane vesicles accumulate free uridine while vesicles of the untransformed line Balb/c 3T3 accumulate either ribose-1-P or UMP or both (the two compounds cochromatograph on our standard system for separating base, nucleoside and nucleotide). The basis for this difference in product is now under study. The preliminary work with the vesicles of mouse and hamster cell lines and the extensive whole cell work showing both variation in the 3T3 line[119,120] and in the mechanism in Novikoff cells[91,93,94,118] further indicate that several mechanisms of uridine uptake and intricate regulation are operative.

b. Thymidine Utilization

Early studies showed that resistance to the thymidine analog, bromodeoxyuridine, also renders mammalian cells incapable of utilization of exogenous thymidine.[120a] Both these conditions are invariably accompanied by the loss of the thymidine kinase which converts thymidine to thymidylate. Thus, growth on bromodeoxyuridine (BUdR) offers a selection system for cells that can no longer use exogenous thymidine and are deficient in thymidine kinase. A counter selection system for the presence of the ability to use exogenous thymidine, sensitivity to BUdR, and presence of thymidine kinase is also available.[100]

This selection system, known as HAT medium (hypoxanthine-amethopterin-thymidine), prevents biosynthesis of the purine ring and of thymidylate from dUMP. Amethopterin, a folate antagonist, blocks the transfer of single carbon units necessary for completion of the purine ring and for methylation of dUMP to form TMP. This selection technique is equally useful for detecting thymidine kinase positive cells from a BUdR-resistant population, and hypoxanthine-guanine phosphoribosyltransferase positive cells from an 8-azaguanine, 6-thioguanine, or 6-mercaptopurine-resistant population.[99] The exquisite sensitivity of the technique has made it the most useful in mammalian somatic cell genetics so far available, and has led to the isolation of revertants in the

10^{-9} reversion frequency category.[88] Thus, it was well established a decade ago that thymidine kinase is an essential element in thymidine incorporation,[100] but a direct relationship between thymidine kinase and thymidine transport into the mammalian cell has only been more recently examined. Schuster and Hare[121] compared uptake and kinase activity levels in several hamster cell lines. Thymidine uptake is correlated with enzyme activity in a number of cell lines possessing thymidine kinase. When studied as a function of temperature and substrate concentration, it was found that lines with high thymidine kinase activity show proportionately greater inhibition of uptake than the cell lines low in thymidine kinase at 5°C relative to 25°C. At 5°C more free thymidine is found inside the cells than TMP. Plagemann has similarly shown[93] that transport can be dissociated from subsequent processes for uridine utilization. Schuster and Hare also observed that free thymidine accumulates in proportion to exogenous substrate concentration, suggesting a passive diffusion component in addition to the kinase in thymidine utilization. It is not clear from this study, however, whether the free thymidine accumulating in the cells at high exogenous concentration or at low temperatures is subsequently used. If thymidine kinase is localized on the membrane inner surface, accumulated thymidine is likely to have bypassed the enzyme. In a thymidine kinase$^-$ mutant, Schuster and Hare detected an uptake rate higher than certain low thymidine kinase lines, though this line could not incorporate the nucleoside after it was taken up. It seems likely that generally greater membrane permeability accounts for this aberrant finding, as the kinetics suggest passive diffusion. The authors offer several models for the role of thymidine kinase uptake and favor a group translocation mechanism or group translocation with the membrane thymidine kinase in equilibrium with a cytoplasmic thymidine kinase. Again, it is most important to ascertain whether the thymidine that does enter the cell unchanged is able to be phosphorylated after accumulation. Thus, if thymidine is phosphorylated only on entrance, only the group translocation mechanism would be of biologic significance despite the ability of thymidine to reach the inside of the cell by either passive diffusion or other transport carriers (e.g., shared specificity with uridine).

In a different approach, Plagemann[122] and Plagemann and Erbe[123] demonstrated that though phosphorylation and uptake are apparently interrelated, the two processes can be dissociated to show that they are likely separate steps. At high thymidine concentrations, uptake by Novikoff cells is observed to consist of a mediated component exhibiting Michealis-Menten kinetics and a passive diffusion component. After treatment of cells with p-chloromercuribenzoate or moderate heat (49.5°C, 5 min), activity of the mediated component is markedly reduced, but phosphorylation of thymidine entering by passive diffusion continues with this thymidylate used for DNA synthesis.[123] A possible explanation for this is that both the p-CMB and heat treatments liberate normally membrane-bound thymidine kinase, and thus convert a group translocation into a two-step reaction at artificially high substrate concentrations – a reaction that would normally not occur in the cells. Though this hypothesis should not be discarded, the simpler two-step mechanism of a facilitated diffusion carrier system with subsequent phosphorylation seems tentatively more acceptable. Since thymidine kinase is in excess in the cells,[123] transport appears to be rate-limiting in thymidine utilization. Uptake is competitively inhibited by persantin and uridine, but whether either of these compounds inhibit without subsequent metabolism (e.g., UMP or UTP may be the direct effector rather than free uridine) was not ascertained. Plagemann and Erbe[123] did, however, characterize the basic kinetics for Novikoff cells: K_m equals to 0.5 μM and V_{max} values with average Q_{10} of 1.8 V_{max} for thymidine kinase in cell-free preparations is 20 times that for cellular thymidine uptake, while K_m for the kinase is 400 times that for transport, further supporting the concept that two separate steps are involved. At external thymidine concentrations greater than 2 μM, passive diffusion was ascertained the major mechanism of thymidine entry into Novikoff cells.

Plagemann and Estensen[124] could further differentiate between uptake and phosphorylation in the presence of cytochalasin B, which inhibited transport without affecting phosphorylation or macromolecular biosynthesis. Cytochalasin B is assumed to possibly alter microfilament structure, but the relation of this effect to transport inhibition is unknown and likely a separate action. Mizel and Wilson[125] found that both colchicine and lumicolchicine inhibit uridine and thymidine trans-

port in several cell lines. Though colchicine does bind to and disrupt microtubule protein, again suggesting a possible relationship between microtubules and transport, the analog lumicolchicine does not, indicating that colchicine inhibition of transport is unrelated to its effect on microtubules.

Thus in hamster cells[121] and in rat hepatoma cells,[122,123] it is observed that thymidine uptake is a mediated process at low substrate levels, but that passive diffusion is significant at higher thymidine concentrations. It appears that phosphorylation is subsequent to uptake in both cases. Whether the mediated or the passive process is operative in vivo likely depends on blood and tissue levels of substrate which would vary with physiological conditions. The importance of the physiological state of the cell to thymidine transport has been recently alluded to by Cunningham and Remo.[126] These authors found that serum stimulation of density-inhibited mouse 3T3 cells is accompanied by a marked increase in thymidine (and deoxycytidine) uptake and in DNA synthesis, without concomitant increases in deoxyadenosine, deoxyguanosine, or orthophosphate utilization. Further, a thymidine kinase-deficient 3T3 subline showed the same increase in transport, which still occurred under conditions in which DNA synthesis was inhibited.

C. Conclusions Concerning Animal Cell Systems

Work on transport has proceeded at a most rapid and impressive rate, almost entirely within the past four years. It is apparent that numerous mechanisms may account for transport of precursors into different cell types, and moreover for uptake of a given precursor at different stages in the cell cycle. Finely tuned and complex regulatory mechanisms are evident and may be essential to growth regulation in mammalian cells, and relevant to the problem of neoplastic transformation. As early as 1969, Cunningham and Pardee[119] recognized major differences in uridine transport in growing vs. "dormant" or contact-inhibited 3T3 cells, while adenosine transport was much less subject to such variation. They further observed[120] even greater differences in uridine uptake when contact-inhibited cells were compared to serum-stimulated cells ("rescued" by fresh serum to undergo one more round of cell division). Differences have also been noted between normal 3T3 cells and a subline transformed by the oncogenic virus, SV40. These observations[119,120] and others led Holley[127] to postulate that lesions in the transport carriers may be responsible for loss of growth control in the cancerous state. To test this hypothesis as well as to better understand the transport processes and their modulating control mechanisms, it seems essential to continue investigation of transport in whole cells and parallel these studies in isolated vesicles. The work with intact cells can be expected to contribute much information concerning the interdigitation of transport and its regulation in the entire economy of cellular growth control, while the vesicle work will contribute to a better understanding of the role of the membrane itself.

IV. NUCLEIC ACID PRECURSOR TRANSPORT IN EXPERIMENTAL SYSTEMS OTHER THAN MAMMALIAN CELLS IN CULTURE AND BACTERIA

A few studies have been carried out in yeast[128–130] and other fungi[131] detailing possible mechanisms of nucleic acid precursor transport. The most notable generalization to be drawn from those studies is that the mechanisms differ from bacterial systems and seem not to involve group translocation, at least for uracil uptake in yeast[128] and adenosine uptake in Streptomyces.[131] The evidence that *Streptomyces antibioticus* utilizes adenosine intact is derived from the fact that adenosine is a direct precursor of the nucleoside antibiotic arabinofuranosyladenine produced by this species. Using $^{15}N/^{14}C$ labeled adenosine Farmer and Suhadolnik[131] ascertained that the isotope ratio in the exogenous adenosine is exactly the same as that in antibiotic synthesized. They therefore concluded that adenosine was taken up without cleavage. Though this is a likely possibility, the evidence does not exclude a membrane mechanism that would take up each moiety individually and reform the nucleoside. A preferential or exclusive utilization of the sugar for nucleotide synthesis has already been observed for *S. typhimurium*.[25] In yeast, the basis for postulating that a group-translocation mechanism for uracil uptake does not exist is Grenson's[128] observation that uptake could be partially restored in a uracil phosphoribosyltransferase deficient strain if appropriately starved.[130] Starvation results in restoration of only ~ 3% of the uptake

activity, yet even in *E. coli* 2 to 3% of the parental uptake activity has been observed in a uracil PRT'ase deficient mutant (Figure 8), so that the small residual activity may represent cross-specificity with the orotate uptake system. Of course, there is no reason to postulate a group translocation mechanism in yeast simply because it exists in enteric bacteria. Recent studies from Grenson's laboratory have also shown that in both *Saccharomyces* and in *Candida*, a common transport system exists for cytosine, adenine, and hypoxanthine which seems not to involve group translocations.[129]

SUMMARY

An attempt has been made to present some of the major recent work in the field of purine and pyrimidine base and nucleoside transport. In bacteria, the emphasis has been placed on developing an appreciation for the role of the periplasm in the processing of these metabolites prior to transport via group translocation enzyme-permease type systems. In animal cells, emphasis has been on the newness of the work and the complexity of mechanisms that seem operative. Further research to illuminate basic transport mechanisms is enthusiastically anticipated, particularly since it may directly relate to patterns of growth control in mammalian cells.

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GLOSSARY OF ABBREVIATIONS

PRPP – 5-phosphoribosyl-1-pyrophosphate

PEP – phosphoenolpyruvate

PRT'ase – phosphoribosyltransferase

ppGpp – 5-pyrophosphorylguanosine-3 pyrophosphate

DNP – 2,4 dinitrophenol

CCCP – carbonylcyanide m-chlorophenylhydrazone

D-LDH – D-Lactic acid dehydrogenase

DCCD – dicyclohexylcarbodimide

HG PRT'ase – Hypoxanthine-guanine phosphoribosyltransferase

A PRT'ase – Adenine phosphoribosyltransferase

PM – plasma membrane

ER – endoplasmic reticulum

R1P – ribose-1-phosphate

AICAR – amino imidazolecarboxamideribotide

N – em

N – ethylmaleimide

REFERENCES

1. **Roberts, R. B., Abelson, R. M., Cowie, D. B., Bolton, E. T., and Britton, R. J.,** Studies of biosynthesis in *Escherichia coli,* Carnegie Institution of Washington, Publication No. 607, 1955.
2. **Berlin, R. and Stadtman, E. R.,** A possible role of purine nucleotide pyrophosphorylases in the regulation of purine uptake by *Bacillus subtilis, J. Biol. Chem.,* 241, 2679, 1966.
3. **Hochstadt-Ozer, J. and Stadtman, E. R.,** The regulation of purine utilization in bacteria. I. Purification of adenine phosphoribosyltransferase and control of activity by nucleotides, *J. Biol. Chem.,* 246, 5294, 1971.
4. **Hochstadt-Ozer, J. and Stadtman, E. R.,** The regulation of purine utilization in bacteria. II. Adenine phosphoribosyltransferase in isolated membrane preparations and its role in transport of adenine across the membrane, *J. Biol. Chem.,* 246, 5304, 1971.
5. **Hochstadt-Ozer, J. and Stadtman, E. R.,** The regulation of purine utilization in bacteria. III. The involvement of purine phosphoribosyltransferase in the uptake of adenine and other nucleic acid precursors by intact resting cells, *J. Biol. Chem.,* 246, 5312, 1971.
6. **Hochstadt-Ozer, J.,** The regulation of purine utilization in bacteria. IV. Roles of membrane-localized and pericytoplasmic enzymes in the mechanism of purine nucleoside transport across isolated *E. coli* membranes, *J. Biol. Chem.,* 247, 2419, 1972.
7. **Kundig, W., Ghosh, S., and Roseman, S.,** Phosphate bound to histidine in a protein as an intermediate in a novel phospho-transferase system, *Proc. Natl. Acad. Sci. U.S.A.,* 52, 1067, 1964.
8. **Hochstadt-Ozer, J. and Cashel, M.,** The regulation of purine utilization in bacteria. V. Inhibition of purine phosphoribosyltransferase activity and purine uptake in isolated membrane vesicles by guanosine tetraphosphate, *J. Biol. Chem.,* 247, 7067, 1972.
9. **Magasanik, B.,** Purine and pyrimidine nucleotides, in *The Bacteria,* Vol. III, Gunsalus, I. C. and Stanier, R. Y., Eds., Academic Press, New York, 1962, 295.
10. **Gots, J. S., Benson, C. E., and Shumas, S. R.,** Genetic separation of hypoxanthine and guanine-xanthine phosphoribosyltransferase activities of deletion mutations in *Salmonella typhimurium, J. Bacteriol.,* 112, 910, 1972.
11. **Chou, J. Y. and Martin, R. G.,** Purine phosphoribosyltransferase of *Salmonella typhimurium, J. Bacteriol.,* 112, 1010, 1972.

11a. **Nierlich, D.,** Amino acid control over RNA synthesis: a reevaluation, *Proc. Natl. Acad. Sci. U.S.A.,* 60, 1345, 1968.

12. **Hochstadt-Ozer, J.,** Regulation of purine uptake in bacteria: Purification of hypoxanthine-quanine phosphoribosyltransferase and effect of 5′ nucleotides on activity and on transport, *Bacteriol. Proc.,* 72, 150, 1972.
13. **Miller, R. L., Ramsey, G. A., Krenitsky, T. A., and Elion, G. B.,** Guanine phosphoribosyltransferase from *Escherichia coli,* specificity and properties, *Biochemistry,* 11, 4723, 1972.
14. **Krenitsky, T. A., Neil, S. M., and Miller, R. L.,** Guanine and xanthine phosphoribosyltransfer activities of *Lactobacillus casei* and *Escherichia coli, J. Biol. Chem.,* 245, 2605, 1970.
15. **Arnold, W. J. and Kelley, Wm. N.,** Human hypoxanthine-guanine phosphoribosyltransferase: Purification and subunits structure, *J. Biol. Chem.,* 246, 7398, 1971.
16. **Arnold, W. J. and Kelley, W. N.,** Human hypoxanthine-guanine phosphoribosyltransferase: Purification and properties, *Isr. J. Med. Sci.,* 9, 1082, 1973.
17. **Thakar, J. H. and Kalle, G. P.,** Defective guanine uptake in an 8-azaguanine resistant mutant of *Salmonella typhimurium, J. Bacteriol.,* 95, 458, 1968.
18. **Coggin, J. H., Loosemore, M., and Martin, W. R.,** Metabolism of 6-mercaptopurine by resistant *Escherichia coli* cells, *J. Bacteriol.,* 92, 446, 1966.
19. **Martin, W. R. and Yang, R. R.,** *E. coli* phosphoribosyltransferase activation of 6-mercaptopurine, *Bacteriol. Proc.,* 72, 154, 1972.
20. **Hochstadt, J.,** Cytidine transport across isolated bacterial membrane vesicles from enteric bacteria, *Bacteriol. Proc.,* 73, 191, 1973.
21. **Hochstadt, J. and Rader, R.,** Cytidine and uridine transport by isolated membrane vesicles from *Salmonella typhimurium* LT-2, *Proc. Int. Congr. Biochem.,* 9, 289, 1973.
22. **Bergmann, F., Mahler, A., Burger-Rachamimov, H., and Diller, D.,** Mechanism of uptake of purines by *Pseudomonas aeruginosa, Biochemistry,* 8, 457, 1969.
23. **Munch-Petersen, A.,** On the catabolism of deoxyribonucleosides in cells and cell extracts of *Escherichia coli, Eur. J. Biochem.,* 6, 432, 1968.
24. **Karlström, O.,** Mutants of *Escherichia coli* defective in ribonucleoside and deoxyribonucleoside catabolism, *J. Bacteriol.,* 95, 1069, 1968.
25. **Hoffmeyer, J. and Neuhard, J.,** Metabolism of exogenous purine bases and nucleosides by *Salmonella typhimurium, J. Bacteriol.,* 106, 14, 1971.
26. **Peterson, R. N., Boniface, J., and Koch, A. L.,** Energy requirements, interactions and distinctions in the mechanisms for transport of various nucleosides in *Escherichia coli, Biochim. Biophys. Acta.,* 135, 771, 1967.
27. **Peterson, R. N. and Koch, A. L.,** The relationship of adenosine and inosine transport in *Escherichia coli, Biochim. Biophys. Acta,* 126, 129, 1966.

28. **Zimmerman, E. F. and Magasanik, B.,** Utilization and interconversion of purine bases and ribonucleosides by *Salmonella typhimurium, J. Biol. Chem.*, 239, 293, 1964.
29. **Hammer-Jespersen, K. and Munch-Petersen,** Phosphodeoxyribomutase from *Escherichia coli,*: Purification and some properties, *Eur. J. Biochem.*, 17, 397, 1970.
30. **Magasanik, B. and Karibian, D.,** Purine nucleotide cycles and their metabolic role, *J. Biol. Chem.*, 235, 2672, 1960.
31. **Moyed, H. S. and Magasanik, B.,** The biosynthesis of the imidazole ring of histidine, *J. Biol. Chem.*, 235, 149, 1960.
32. **Komatsu, Y. and Tanaka, K.,** A showdomycin-resistant mutant of *Escherichia coli* K-12 with altered nucleoside transport character, *Biochim. Biophys. Acta,* 288, 390, 1972.
33. **Munch-Petersen, A.,** Thymidine breakdown and thymine uptake in different mutants of *Escherichia coli, Biochim. Biophys. Acta,* 142, 228, 1967.
34. **Neu, H.C. and Heppel, L. A.,** The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts, *J. Biol. Chem.*, 240, 3685, 1965.
35. **Leder, I.,** Interrelated effects of cold shock and osmotic pressure on the permeability of the *Escherichia coli* membrane to permease accumulated substrates, *J. Bacteriol.*, 111, 211, 1972.
36. **Beck, C. F., Ingraham, J. L., Neuhard, J., and Thomassen, E.,** Metabolism of pyrimidines and pyrimidine nucleosides by *Salmonella typhimurium, J. Bacteriol.*, 110, 219, 1972.
37. **Kaback, H. R.,** Bacterial membranes, *Methods in Enzymology,* Vol. XXII, Jakoby, W. B., Ed., 1971, 99.
38. **Osborne, M. J., Gander, J. E., Parise, E., and Carson, J.,** Mechanism of assembly of the outer membrane of *Salmonella typhimurium* isolation and characterization of cytoplasmic and outer membrane, *J. Biol. Chem.*, 247, 3962, 1972.
39. **Grenson, M.,** The utilization of exogenous pyrimidines and the recycling of uridine-5′-phosphate derivatives in *Saccharomyces cerevisiae,* as studied by means of mutants affected in pyrimidine uptake and metabolism, *Eur. J. Biochem.*, 11, 249, 1969.
40. **Pierard, A., Glansdorff, N., and Yashphe, J.,** Mutations affecting uridine monophosphate pyrophosphorylase or the argR gene in *Escherichia coli, Mol. Gen. Genet.*, 118, 235, 1972.
41. **Von Dippe, P. J., Roy-Berman, S., and Visser, B. W.,** Transport of uridine in *Escherichia coli* B and a showdomycin-resistant mutant, *Biochim. Biophys. Acta,* 318, 105, 1973.
42. **Crawford, I., Kornberg, A., and Simms, E. S.,** Conversion of uracil and orotate to uridine 5′-phosphate by enzymes in lactobacilli, *J. Biol. Chem.*, 226, 1093, 1957.
43. **Neuhard, J. and Ingraham, J.,** Mutants of *Salmonella typhimurium* requiring cytidine for growth, *J. Bacteriol.*, 95, 2431, 1968.
44. **Kaback, H. R.,** The role of the phosphoenolpyruvate phosphosphotransferase system in the transport of sugars by isolated membrane preparations of *Escherichia coli, J. Biol. Chem.*, 243, 3711, 1968.
45. **Sercarz, E. E. and Gorini, L.,** Different contribution of exogenous and endogenous arginine to repressor formation, *J. Mol. Biol.*, 8, 254, 1964.
46. **Kammen, H. O.,** Thymine metabolism in *Escherichia coli* I. Factors involved in utilization of exogenous thymine, *Biochim. Biophys. Acta,* 134, 301, 1967.
47. **Rachmeler, M., Gerhart, J., and Rosner, J.,** Limited thymidine uptake in *Escherichia coli* due to an inducible thymidine phosphorylase, *Biochim. Biophys. Acta,* 49, 222, 1961.
48. **Breitman, T. R. and Bradford, R. M.,** The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation, *Biochem. Biophys. Res. Comm.*, 17, 786, 1964.
49. **Boyce, R. P. and Setlow, R. B.,** A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of *Escherichia coli, Biochim. Biophys, Acta,* 61, 618, 1972.
50. **Razzel, W. E. and Casshyap, P.,** Substrate specificity and induction of thymidine phosphorylase in *Escherichia coli, J. Biol. Chem.*, 239, 1789, 1964.
51. **Crawford, L.,** Thymine metabolism in strains of *Escherichia coli, Biochim. Biophys. Acta,* 30, 428, 1958.
51a. **Jensen, K. F., Leer, J. C., and Nygaard, P.,** Thymine utilization in *Escherichia coli* K_{12} on the role of deoxyribose-1-phosphate and thymidine phosphorylase, *Eur. J. Biochem.*, 40, 345, 1973.
52. **Stock, J. and Roseman, S.,** Evidence for two solute pools in *Salmonella typhimurium, Fed. Proc.*, abstr., 32, 517, 1973.
53. **Roy-Berman, J. and Visser, D. W.,** Transport studies of showdomycin nucleosides and sugars in *Escherichia coli* B and in showdomycin-resistant mutants, *Biochim. Biophys. Acta,* 282, 383, 1972.
54. **Darnall, K. R., Townsend, L. B., and Robins, R. K.,** The structure of showdomycin, a novel carbon-linked nucleoside antibiotic related to uridine, *Proc. Natl. Acad. Sci. U.S.A.*, 57, 548, 1967.
55. **Hochstadt, J. and Rader, R. R.,** Cytidine and uridine transport by isolated membrane vesicles from *Salmonella typhimurium* LT-2, *Int. Congr. Bacteriol.*, 1, 80, 1973.
56. **Kaback, H. R.,** Transport across isolated bacterial cytoplasmic membranes, *Biochim. Biophys. Acta,* 265, 367, 1972.
57. **Komatsu, Y. and Tanaka, K.,** Deoxycytidine uptake by isolated membrane vesicles from *Escherichia coli* K12, *Biochim. Biophys. Acta,* 311, 496, 1973.
58. **Nygaard, P.,** Nucleoside-catabolizing enzymes in *Salmonella typhimurium:* Induction by ribonucleosides, *Eur. J. Biochem.*, 36, 267, 1973.

59. **Bhattacharyya, P., Epstein, W., and Silver, S.,** Valinomycin-induced uptake of potassium in membrane vesicles from *Escherichia coli, Proc. Natl. Acad. Sci. U.S.A.*, 68, 1488, 1971.
60. **Thompson, J. and MacLeod, R. A.,** Electron-donor energized transport of -aminoisobutyric acid (AIB) and K+ into intact cells of a marine pseudomonad, *Abstr. Meeting Am. Soc. Microbiol.*, 73, 190, 1973.
61. **Hong, J. S. and Kaback, H. R.,** Mutants of *Salmonella typhimurium* and *Escherichia coli* pleiotropically defective in active transport, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 3336, 1972.
62. **Simoni, R. D. and Shallenberger, M. K.,** Coupling of energy to active transport of amino acids in *Escherichia coli, Proc. Natl. Acad. Sci. U.S.A.*, 69, 2663, 1972.
63. **Tabor, H. and Tabor, C. W.,** Partial separation of two pools of arginine in *Escherichia coli;* preferential use of exogenous rather than endogenous arginine for the biosynthesis of 4-diaminobutane, *J. Biol. Chem.*, 244, 6383, 1969.
63a. **Raue, H. A. and Cashel, M.,** Regulation of RNA synthesis in *Escherichia coli.* I. Characterization of cells subjected to simultaneous temperature and osmotic shock, *Biochim. Biophys. Acta*, 312, 722, 1973.
64. **Hawkins, R. A. and Berlin, R. D.,** Purine transport in polymorphonuclear leukocytes, *Biochim. Biophys. Acta*, 173, 324, 1969.
65. **Berlin, R. D.,** Specificities of transport systems and enzymes, *Science*, 168, 1539, 1970.
66. **Taube, R. A. and Berlin, R. D.,** Membrane transport of nucleosides in rabbit polymorphonuclear leukocytes, *Biochim. Biophys. Acta*, 255, 6, 1972.
67. **Tsan, M. F. and Berlin, R. D.,** Membrane transport in the rabbit alveolar macrophage. The specificity and characteristics of amino acid transport systems, *Biochim. Biophys. Acta*, 241, 155, 1971.
68. **Tsan, M. F. and Berlin, R. D.,** Effect of phagocytosis on membrane transport of nonelectrolytes, *J. Exp. Med.*, 134, 1016, 1971.
69. **Berlin, R. D.,** Temperature dependence of nucleoside membrane transport in rabbit alveolar macrophages and polymorphonuclear leukocytes, *J. Biol. Chem.*, 248, 4724, 1973.
70. **Whittam, R.,** The high permeability of human red cells to adenine and hypoxanthine and their ribosides, *J. Physiol.* (Lond.), 154, 614, 1960.
71. **Jacquez, J. A. and Ginsberg, F.,** Permeability of Ehrlich ascites cells to adenine, *Proc. Soc. Exp. Biol. Med.*, 105, 478, 1960.
72. **Sanford, J. B. and Rosenberg, M. D.,** Nucleotide phosphohydrolase activities of the plasma membranes of embryonic chick liver cells, *Biochim. Biophys. Acta*, 288, 333, 1972.
73. **Kaback, H. R.,** Transport, *Annu. Rev. Biochem.*, 39, 561, 1970.
74. **Berlin, R. D. and Ukena, T. E.,** Effects of colchicine and vinblastine on the agglutination of polymorphonuclear leucocytes by concanavalin A, *Nat. New Biol.*, 238, 120, 1972.
75. **Yin, H. H., Ukena, T. E., and Berlin, R. D.,** Effects of colchicine colcemid and vinblastine on the agglutination, by concanavalin A, of transformed cells, *Science*, 178, 867, 1972.
76. **Ukena, T. E. and Berlin, R. D.,** Effect of colchicine and vinblastine on the topographical separation of membrane functions, *J. Exp. Med.*, 136, 1, 1972.
77. **Hochstadt, J. and Dowd, D.,** Uptake of nucleosides and bases of purified membrane vesicles from mammalian cells in culture, *Fed. Proc.*, 32, 636, 1973.
78. **Li, C. and Hochstadt, J.,** Transport of nucleosides in membrane vesicles from mammalian cells, *Proc. Am. Soc. Cell Biol.*, 13, 193a, 1973.
79. **Wallach, D. F. H. and Kamat, V. B.,** Preparation of plasma-membrane fragments from mouse ascites tumor cells, *Methods in Enzymology*, Vol. VIII, Ginsburg, V., and Neufeld, E., Eds., Academic Press, N.Y., 1966, 164.
80. **Berezney, R., Macaulay, L. K., and Crane, F. L.,** The purification and biochemical characterization of bovine liver nuclear membranes, *J. Biol. Chem.*, 247, 5549, 1972.
81. **Earl, D. C. N. and Korner, A.,** The isolation and properties of cardiac ribosomes and polysomes, *Biochem. J.*, 94, 721, 1965.
82. **Gianetto, R. and deDuve, C.,** Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles, *Biochem. J.*, 59, 433, 1955.
83. **Morre, D. J.,** Isolation of golgi apparatus, *Meth. Enzymol.*, XXII, Jakoby, W. B. Ed., Academic Press, N.Y., 1971, 130.
84. **Ernster, L., Siekevitz, P., and Palade, G. E.,** Enzyme-structure relationships in the endoplasmic reticulum of rat liver, *J. Cell Biol.*, 15, 541, 1962.
85. **Ozer, J. H(ochstadt) and Wallach, D. F. H.,** H-2 components and cellular membranes: Distinctions between plasma membrane and endoplasmic reticulum governed by the *H-2* region in the mouse, *Transplantation*, 5, 652, 1967.
86. **Carter, J. E., Avruch, J., and Martin, D. B.,** Glucose transport in plasma membrane vesicles from rat adipose tissue, *J. Biol. Chem.*, 247, 2682, 1972.
86a. **Wallach, D. F. H., Kamat, V. B., and Gail, M. H.,** Physicochemical differences between fragments of plasma membrane and endoplasmic reticulum, *J. Cell Biol.*, 30, 601, 1966.
87. **Rosenbloom, F. M., Kelley, W. N., Henderson, J. F., and Seegmiller, J. E.,** Lyon hypothesis and x-linked disease, *Lancet*, 2, 305, 1967.
88. **McBride, W. and Ozer, H. L.,** Transfer of genetic information by purified metaphase chromosomes, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 1258, 1973.

89. **Gotto, A. M., Meikle, A. W., and Touster, O.,** Nucleoside metabolism in Ehrlich ascites tumor cells: Phosphorolysis of purine nucleosides, *Biochim. Biophys. Acta,* 80, 552, 1964.
90. **Steck, T. L., Nakata, Y., and Bader, J. P.,** The uptake of nucleosides by cells in culture. I. Inhibition by heterologous nucleosides, *Biochim. Biophys. Acta,* 190, 237, 1969.
91. **Plagemann, P. G. W.,** Nucleoside transport by Novikoff rat hepatoma cells growing in suspension culture: Specificity and mechanism of transport reactions and relationship to nucleoside incorporation into nucleic acids, *Biochim. Biophys. Acta,* 233, 688, 1971.
92. **Crabtree, G. W. C. and Henderson, J. F.,** Pathways of purine ribonucleotide catabolism in Ehrlich ascites tumor cells *in vitro, Can. J. Biochem.*, 49, 959, 1971.
93. **Plagemann, P. G. W.,** Nucleotide pools of Novikoff rat hepatoma cells growing in suspension culture. I. Kinetics of incorporation of nucleosides into nucleotide pools and pool sizes during growth cycle, *J. Cell Physiol.*, 77, 213, 1971.
94. **Plagemann, P. G. W. and Erbe, J.,** Nucleotide pools of Novikoff rat hepatoma cells growing in suspension culture. IV. Nucleoside transport in cells depleted of nucleotides by treatment with KCN, *J. Cell Biol.*, 52, 131, 1972.
95. **Plagemann, P. G. W.,** Nucleotide pools in Novikoff rat hepatoma cells growing in suspension culture. III. Effects of nucleosides in medium on levels of nucleotides in separate nucleotide pools for nuclear and cytoplasmic RNA synthesis, *J. Cell Biol.*, 52, 131, 1972.
96. **Plagemann, P. G. W. and Erbe, J.,** Transport and metabolism of glucosamine by cultured Novikoff rat hepatoma cells and effects on nucleotide pools, *Cancer Res.*, 33, 482, 1973.
97. **Levine, E.,** A simplified method for the detection of mycoplasm, *Methods in Cell Biology,* Vol. 8, Prescott, D., Ed., 1974.
98. **Cunningham, D. and Pardee, A. B.,** Transport charges rapidly initiated by serum addition to "contact inhibited" 3T3 cells, *Proc. Natl. Acad. Sci. U.S.A.*, 64, 1049, 1969.
99. **Ozer, H. L.,** Purine pyrophosphorylase as a selective genetic marker in a mouse lymphoma, P338, in cell culture, *J. Cell Physiol.*, 68, 61, 1966.
100. **Littlefield, J. W. and Basilico, C.,** Infection of thymidine kinase-deficient BHK cells with polyoma virus, *Nature,* 211, 250, 1966.
101. **Littlefield, J. W.,** Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants, *Science,* 145, 709, 1964.
102. **Morrow, J., Colofiore, J., and Rintoul, D.,** Azaguanine resistant hamster cell lines not deficient in hypoxanthine-guanine phosphoribosyltransferase, *J. Cell Physiol.*, 81, 97, 1973.
103. **Beaudet, A. L., Roufa, D. J., and Caskey, C. T.,** Mutations affecting the structure of hypoxanthine: Guanine phosphoribosyltransferase in cultured Chinese hamster cells, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 320, 1973.
104. **Arnold, W. J., Meade, J. C., and Kelley, W. N.,** Hypoxanthine-guanine phosphoribosyltransferase: Characteristics of the mutant enzyme in erythrocytes from patients with the Lesch-Nyhan syndrome, *J. Clin. Invest.*, 51, 1805, 1972.
105. **Rosenbloom, F. M., Kelley, W. N., Miller, J., Henderson, J. F., and Seegmiller, J. E.,** Inherited disorder of purine metabolism. Correlation between central nervous system dysfunction and biochemical defects, *J. Am. Med. Assoc.*, 202, 175, 1967.
106. **Murray, A. W.,** The biological significance of purine salvage, *Annu. Rev. Biochem.*, 40, 811, 1971.
107. **Raivio, K. O. and Seegmiller, J. E.,** Genetic diseases of metabolism, *Annu. Rev. Biochem.*, 41, 543, 1972.
108. **Fox, I. H. and Kelley, W. N.,** Phosphoribosyl pyrophosphate in man: Biochemical and clinical significance, *Ann. Intern. Med.*, 74, 424, 1971.
109. **Adye, J. C. and Gots, J. S.,** Further studies on genetically altered purine nucleotide pyrophosphorylases on *Salmonella, Biochim. Biophys. Acta,* 118, 344, 1966.
109a. **Benke, P. J., Herrick, N., and Herbert, A.,** Transport of hypoxanthine in fibroblasts with normal and mutant hypoxanthine-guanine phosphoribosyltransferase, *Biochem. Med.*, 8, 309, 1973.
110. **Quinlan, D. and Hochstadt, J.,** Transport of nucleosides by plasma membrane vesicles isolated from Balb/c 3T3 and Balb/c SV-3T3 cells, *Fed. Proc.*, 34, 1974 (in press).
111. **Paterson, A. R. P. and Oliver, J. M.,** Nucleoside transport. II. Inhibition by p-nitrobenzylthioguanosine and related compounds, *Can. J. Biochem.*, 49, 271, 1971.
112. **Gabrio, B. W., Hennessey, M., Thomasson, J., and Finch, C. A.,** Erythrocyte preservation. IV. *In vitro* reversibility of the storage lesion, *J. Biol. Chem.*, 215, 357, 1955.
112a. **Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H., and Abe, T.,** Effect of inosine and adenine on adenosine triphosphate regeneration and shape transformation in long-stored erythrocytes, *Biochim. Biophys. Acta,* 31, 564, 1959.
113. **Schneider, E. I., Epstein, C. S., Epstein, W. L., Betlach, M., and Abbohalbasch, G.,** Detection of mycoplasma contamination in cultured human fibroblasts comparison of biochemical and microbiological techniques, *Exp. Cell Res.*, 79, 343, 1973.
114. **Itzhaki, S.,** Regulation of uracil utilization for RNA synthesis in Ehrlich ascites tumor cells by glucose metabolism, *Life Sci.*, 11, 649, 1972.
115. **Miyamoto, M. and Terayama, H.,** Tumor cell sap factor stimulating orotate incorporation into nucleic acids in ascites hepatoma cells, *Biochim. Biophys. Acta,* 272, 612, 1972.

116. **Littlefield, J. W. and Basilico, C. A.,** Infection of thymidine kinase-deficient BHK cells with polyoma virus, *Nature,* 211, 250, 1966.
117. **Lieber, M. M., Benveniste, R. E., Livingston, D. M., and Todaro, G. J.,** Mammalian cells in culture frequently release type C viruses, *Science,* 182, 56, 1973.
118. **Plagemann, P. G. W. and Roth, M. F.,** Permeation as the rate limiting step in phosphorylation of uridine and choline and their incorporation in macromolecules by Novikoff hepatoma cells. Competitive inhibition by phenethyl alcohol, persantin and adenosine, *Biochemistry,* 8, 4782, 1969.
119. **Cunningham, D. D. and Pardee, B.,** Transport changes rapidly initiated by serum addition to "contact inhibited" 3T3 cells, *Proc. Natl. Acad. Sci. U.S.A.,* 64, 1049, 1969.
120. **Cunningham, D. D. and Pardee, B.,** Transport and phospholipid changes related to control of growth in 3T3 cells, in Ciba Foundation Symposium on *Growth Control in Animal Cells Culture,* Wolstenholme, G. G. W., and Knight, J., Eds., Churchill-Livingstone, Edinburgh and London, 207, 1971.
120a. **Dubbs, D. R. and Kit, S.,** Effect of halogenated pyrimidines and thymidine on growth of L-cells and a subline lacking thymidine kinase, *Exp. Cell Res.,* 33, 19, 1964.
121. **Schuster, G. S. and Hare, J. D.,** The role of phosphorylation in the uptake of thymidine in mammalian cells, *In Vitro,* 6, 427, 1971.
122. **Plagemann, P. G. W.,** Effects of phenethyl alcohol on transport reactions, nucleotide pools and macromolecular synthesis in Novikoff rat hepatoma cells growing in suspension culture, *J. Cell Physiol.,* 75, 315, 1970.
123. **Plagemann, P. G. W. and Erbe, J.,** Thymidine transport by cultured Novikoff hepatoma cells and uptake by simple diffusion and relationship to incorporation into deoxyribonucleic acid, *J. Cell Biol.,* 55, 161, 1972.
124. **Plagemann, P. G. W. and Estensen, R. D.,** Cytochalasin, B. VI. Competitive inhibition of nucleoside transport by cultured Novikoff rat hepatoma cells, *J. Cell Biol.,* 55, 179 , 1972.
125. **Mizel, S. B. and Wilson, L.,** Nucleoside transport in mammalian cells. Inhibition by colchicine, *Biochemistry,* 11, 2573, 1972.
126. **Cunningham, D. and Remo, R. A.,** Specific increase in pyrimidine deoxynucleoside transport at the time of deoxyribonucleic acid synthesis in 3T3 mouse cells, *J. Biol. Chem.,* 248, 6282, 1973.
127. **Holley, R. W.,** A unifying hypothesis concerning the nature of malignant growth, *Proc. Natl. Acad. Sci. U.S.A.,* 69, 2840, 1972.
128. **Grenson, M.,** The utilization of exogenous pyrimidines and the recycling of uridine-5′-phosphate derivatives in *Saccharomyces cerevisiae,* as studies by means of mutants affected in pyrimidine uptake and metabolism, *Eur. J. Biochem.,* 11, 249, 1969.
129. **Polak, A. and Grenson, M.,** Evidence for a common transport system for cytosine, adenine, and hypoxanthine in *Saccharomyces cerevisiae* and *Candida albicans, Eur. J. Biochem.,* 32, 276, 1973.
130. **Grenson, M.,** Specificity and regulation of the uptake and retention of amino acids and pyrimidines in yeast, in *Genetics of Industrial Microorganisms,* Vol. 2, *Actinomycites and Fungi,* Proceedings of a Symposium held on Genetics of Industrial Microorganism, in Prague, August 23, 1970, 179.
131. **Farmer, P. B. and Suhadolnik, R. J.,** Nucleoside antibiotics: Biosynthesis of arabinofuranosulfadenine by *Streptomyces antibioticus, J. Biol. Chem.,* 248, 1844, 1973.