Mechanism of Purine Nucleoside Handling and Transport in Isolated Membrane Vesicles from Polyoma Transformed BHK/21 Cells†

Diana J. Dowd,‡ Dennis C. Quinlan,§ and Joy Hochstadt*,¶

ABSTRACT: Inosine uptake into membrane vesicles prepared from baby hamster kidney (BHK) cells appears to proceed by two distinct mechanisms. One mechanism results in the accumulation of ribose 1-phosphate (ribose-1-P) while hypoxanthine is left in the medium. It has a low K_m and a high initial rate. Since inosine is cleaved, it is a purine nucleoside phosphorylase dependent step. The second mechanism is purine nucleoside phosphorylase independent and results in intravesicular accumulation of intact inosine. The resultant inosine is metabolized by a purine nucleoside phosphorylase activity, which may or may not be the same as that used in the first mechanism which resulted in extravesicular hypoxanthine, leading to hypoxanthine and additional ribose-1-P. The initial rate and K_m of the two mechanisms are distinct and the phosphorylase-dependent mechanism is more sensitive to inhibition by *N*-ethylmaleimide and adenine. Both the mechanisms show the same steady-state level of substrate accumulation. Adenosine is also taken up by the vesicles but kinetic data indicate that it is unlikely that adenosine and inosine are taken up by the same vesicles. Data are provided for subfractionation of the plasma membrane with respect to enzyme function as well as transport function. The paper concludes that there are a variety of nucleoside transport mechanisms available to mammalian cells and that a single cell line can exhibit alternative transport modes.

Previous studies from this laboratory have shown that, when inosine interacts with isolated plasma membrane vesicles from cultured mouse fibroblast cells grown to high cell density, the predominant intravesicular transport product is ribose-1-P[1] (Li and Hochstadt, 1976a,b; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976). The mechanism involves membrane-localized purine nucleoside phosphorylase acting in a group translocation reaction during which hypoxanthine is released on the exterior membrane surface while the ribose moiety is phosphorylated in the process of being transported across the membrane.

In contrast, in polyoma transformed baby hamster kidney (BHK) cells there exist two mechanisms of inosine handling: one, similar to that we have described for the mouse fibroblast cells, in which group translocation occurs and depends on a transmembranal purine nucleoside phosphorylase and for which internal inosine is not a substrate (Hochstadt and Quinlan, 1976; Li and Hochstadt, 1976b) and a second mechanism which involves the uptake of intact inosine. The intact, intravesicular inosine remains intact or is degraded to hypoxanthine and ribose-1-P depending on the state of purification of the vesicles.

Experimental Procedure

Cell Culture. The baby hamster cell line transformed by polyoma virus (BHK21/C13 H6) was obtained from Dr. Albert Murray, Worcester Foundation for Experimental Biology. Dulbecco's modified Eagle's medium (DME) and baby hamster kidney (BHK-21) medium were obtained from Grand Island Biological Co. (GIBCO). Trypsin–EDTA and tryptose phosphate broth (Casein Hydrolysate Peptone No. 40) were also purchased from GIBCO. Fetal calf serum was purchased from both GIBCO and Flow Labs.

Cells were grown in confluence in 720-cm^2 roller bottles. Either DME with 10% fetal calf serum (FCS) or BHK-21 supplemented with 10% FCS and 0.2% casein hydrolysate peptone No. 40 were used to grow the cells and they were fed every 2 to 3 days. The cells were always fed within the 24-h period prior to harvesting—this facilitated cell rupture without concomitant nuclear rupture.

Cell cultures were routinely tested for absence of mycoplasma by aerobic and anaerobic culture performed by HEM Research, Baltimore, Md., and Flow Laboratories, Rockville, Md.

Other Materials. [8-^{14}C]Hypoxanthine, [8-^{14}C]adenosine, [U-^{14}C]adenosine, [8-^{14}C]inosine, and [U-^{14}C]inosine were purchased from Amersham/Searle. The specific radioactivity of the 8-^{14}C-labeled compounds was 55–61 mCi/mmol as purchased; the specific radioactivity of the U-^{14}C-labeled compounds was 500–600 mCi/mmol as purchased.

[U-^{14}C]Adenosine 5′-monophosphate was purchased from New England Nuclear, Inc., Dextran 110 from Pharmacia, and other chemicals were purchased from Sigma and Calbiochem.

Membrane Preparation. Cell monolayers were washed twice in phosphate-buffered saline (PBS), scraped (with a modified windshield wiper), and centrifuged in PBS. The cells were then recentrifuged and resuspended in 0.25 M sucrose, 5 mM

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‡ Present address: Department of Biology, State University of New York, Albany, New York 12222.

§ Present address: Department of Biology, West Virginia University, Morgantown, West Virginia 26506.

¶ Present address: Department of Microbiology, New York Medical College, Valhalla, New York 10595.

[1] Abbreviations used: ribose-1-P, ribose 1-phosphate; BHK, baby hamster kidney; EDTA, ethylenediaminetetraacetic acid; DME, modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; P_i, inorganic phosphate.

Tris-HCl (pH 7.4), 0.2 mM $MgSO_4$ and subjected to nitrogen cavitation at 700–800 psi for 10–20 min, according to the method of Wallach and Kamat (1966). Cell breakage by the cavitation procedure was monitored using phase contrast microscopy. After return of the cells to atmospheric pressure, the cell homogenate was made 1 mM with respect to K-EDTA (pH 6.9). All procedures were performed at 0–4 °C.

Differential centrifugation yielded a nuclear pellet (also containing some unbroken cells) and a mitochondrial–lysosomal pellet. The resulting supernatant fluid was centrifuged at 100 000*g*. The pellet from this latter centrifugation was resuspended in 0.25 M sucrose, 1 mM Tris–HCl (pH 7.4) and was composed of a mixture of plasma membrane and endoplasmic reticulum vesicles. This mixed vesicle preparation was subsequently assayed for transport capability. For a detailed description of our modification of the Wallach and Kamat procedure and our results of fractionation, see Hochstadt et al. (1975).

After it had been ascertained that the mixed vesicles are transport-competent [with BHK membranes not all batches were transport-active, unlike mouse fibroblast cell membranes (Li and Hochstadt, 1971a,b; Quinlan and Hochstadt, 1976)], the plasma membrane and endoplasmic reticulum components were separated on discontinuous dextran 110 (110 000 molecular weight) gradients according to the method of Wallach and Kamat (1966), as described by Hochstadt et al. (1975).

Marker Enzyme Assays. Membrane purity was determined using the following marker enzymes. [All procedures are described in detail by Hochstadt et al. (1975)]. Endoplasmic reticulum was monitored by assaying for NADH dehydrogenase activity; succinate dehydrogenase was used to monitor mitochondria (King, 1967); 5′-nucleotidase (Weaver and Boyle, 1969) and (Na^+, K^+)ATPase (Wallach and Kamat, 1966) were used as marker enzymes for plasma membrane.

Transport Assay. Transport activity was determined using a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.4), 0.12 M sucrose, 0.2 to 0.5 mg of membrane vesicle protein, and radioactively labeled substrate in a total volume of 100 μL. After the vesicles had been preincubated for 10 min at 37 °C, the reaction was initiated by adding the labeled substrate (100 μM, unless otherwise noted). The reaction was stopped by diluting with 1 mL of warm 0.8 M NaCl containing 10 mM uridine and collecting the vesicles on 0.3 μm (pore size) nitrocellulose filters under suction. Uridine was used to lower the background of nucleoside radioactivity which nonspecifically bound to the filters. The filters were washed twice with salt/uridine, dried, and counted in a gas flow spectrometer (Nuclear Chicago) operating at 17% efficiency. A control sample was prepared by dilution of the membranes in the uridine/salt solution prior to addition of the substrate followed by immediate filtration. The control sample usually gave a value of 100–200 cpm. The experimental values from which this was subtracted were in the range of 1000–8000 cpm. The dilution in highly hypertonic salt solution as a means of terminating the reaction and minimizing exit has been a well-established procedure for vesicle transport assays with both bacterial and animal cell membranes (see Hochstadt et al., 1975). Uptake is calculated in pmol min^{-1} (mg of membrane protein)$^{-1}$. In order to characterize the transport products, the filters were treated so as to release vesicle contents which were then chromatographed as described by Hochstadt et al. (1975), using Eastman chromagram thin-layer sheets. Chromatography solvents and radioautographic identification of compounds were as described in Rader and Hochstadt (1976).

Gradient Separation of Plasma Membrane and Endoplasmic Reticulum. The mixed vesicle population could be separated into plasma membrane and endoplasmic reticulum components using discontinuous dextran 110 gradients. The mixed vesicles (designated unwashed mixed vesicles) were diluted in 10 volumes of 10 mM Tris-HCl (pH 8.0) to remove adsorbed protein. The vesicles were collected by centrifugation at >100 000*g* for 30 min and then lysed transiently by resuspending and diluting in 10 or more volumes of 1 mM Tris-HCl (pH 8.0) to release trapped intravesicular protein. About one-third of the total protein was lost at each step. The membranes were collected by centrifugation at >100 000*g* for 30 min and were then resuspended in 1 mM Tris-HCl (pH 8.0) and dialyzed against 200–500 volumes of 1 mM Tris-HCl (pH 8.0) plus 0.2 mM $MgSO_4$ for 1.5–2 h (designated washed mixed vesicles). The dialyzed vesicle suspension was layered over a discontinuous 10%, 16%, and 23% dextran 110 gradient, made up in 1 mM Tris (pH 8.0) plus 0.2 mM $MgSO_4$. Centrifugation was carried out at 30 000*g* for 12–13 h. Opaque bands of membrane formed at each dextran barrier. The bands were removed from the gradient with a canula-fitted syringe and washed twice in 1 mM Tris-HCl (pH 8.0) plus 0.25 M sucrose. The endoplasmic reticulum, which was found with the 23% dextran fraction after centrifugation, was also resuspended in this Tris–sucrose solution and saved for enzyme-marker analysis. The vesicles were stored at −79 °C in 1 mM Tris-HCl (pH 7.4) plus 0.25 M sucrose until assayed for marker enzymes and transport capability.

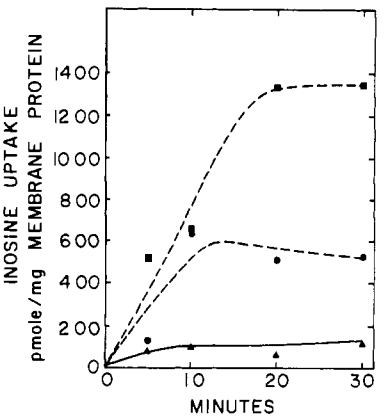

FIGURE 1: Uptake of inosine by membrane fractions from BHK cells. Membrane preparation and transport activity determinations were as described under Experimental Procedure. The membranes were preincubated at 37 °C for 10 min and then exposed to 100 μM [U-^{14}C]inosine for the times indicated. Uptake by unwashed mixed vesicles (▲); uptake by washed and dialyzed mixed vesicle population (●), and by purified plasma membrane material opaque band with lowest buoyant density after separation on dextran 110 (■).

Results

Increase in Specific Activity of Transport with Membrane Purification. Figure 1 shows the increase in specific activity of uptake, using [U-^{14}C]inosine as transport substrate, for unwashed mixed vesicles, for washed and transiently lysed mixed vesicles, and for purified plasma membranes. The increase in specific activity at each step is inversely proportional to the protein recovered in that fractionation step. This suggests that only the plasma membrane is responsible for transport activity and that inosine transport activity is not destroyed by the manipulations required for the final steps of fractionation. The data also suggest that the use of some transport systems as plasma membrane markers, in vesicle systems, may be superior to certain enzyme markers presently in use. This may be particularly true for BHK cells since inosine transport ac-

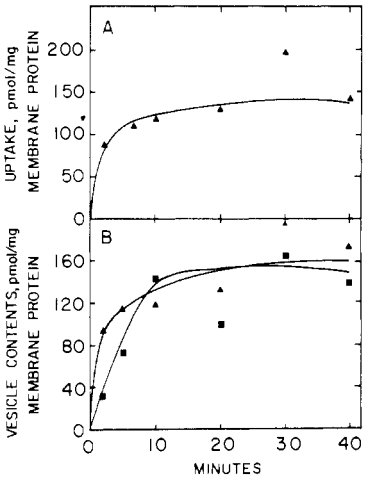

FIGURE 2: Uptake and chromatography of vesicle contents after exposure to [U-^{14}C]inosine. Mixed vesicles were preincubated for 10 min at 37 °C and exposed to 100 μM [U-^{14}C]inosine, and the reaction was allowed to continue for the times indicated. Total accumulation of radioactivity by the vesicles is shown in panel A. The filters were then eluted in aliquots of boiling water, and the eluates lyophilized, resuspended in water, and chromatographed. The only two radioactive spots detected were ribose-1-P (▲) and hypoxanthine (■), and their accumulation by the membrane vesicles is shown in panel B.

TABLE I: Kinetic Constants for Purine Nucleoside Accumulation.

Activity	Apparent K_m (μM)
Uptake of total radioactivity	64
Accumulation of ribose 1-phosphate	33
Accumulation of hypoxanthine	133

tivity is fully recovered from the final gradient steps while the marker enzyme activities are not (e.g., recovery of 5′-nucleotidase ≤86%). Subsequent data indicate that there are two mechanisms of inosine uptake. Since the experiment presented here regarding the specific activity of transport during the purification procedure did not take each process into account separately, the possibility exists that an increase in one or the other mechanism or some unequal combination of the two is responsible for the increased specific activity.

Kinetics of Inosine Transport. The time course of uptake of total radioactivity after exposure to [U-^{14}C]inosine by the unwashed mixed membrane vesicle population is shown in Figure 2A. Figure 2B shows that, when the intravesicular transport products are subjected to chromatography, ribose-1-P and hypoxanthine are observed in the vesicles and attain the same steady-state levels. Inosine, however, was not observed in the vesicles. The ribose-1-P and hypoxanthine could have arisen by cleavage of inosine before, during, or after uptake. Such initial metabolism would be mediated by a purine nucleoside phosphorylase enzyme, which would catalyze the following reaction: inosine + $P_i \rightleftharpoons$ hypoxanthine + ribose-1-P. Ribose-1-P accumulates more rapidly at early times than does the hypoxanthine, which suggests that the hypoxanthine might be leaking out. If this were the case, however, then at steady-state less hypoxanthine should also be found in vesicles, which is not the case.

If separate uptake mechanisms are responsible for the accumulation of hypoxanthine and ribose-1-P, then the same steady-states with different initial uptake rates are possible. Distinct K_m values for the two processes would also be possible. Figure 3 and Table I show uptake kinetics for total radioactivity uptake ribose-1-P accumulation and hypoxanthine accumulation. Though some scatter is always observed in these assays, the K_ms were confirmed by numerous repeat experiments. Also, the fourfold difference between the K_m for hypoxanthine accumulation and ribose-1-P accumulation is quite reproducible and significant.

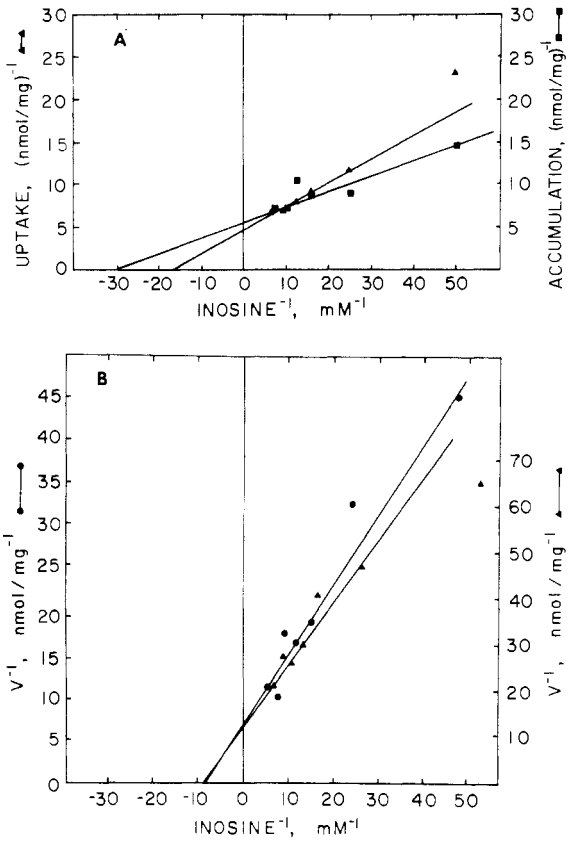

FIGURE 3: Kinetics of inosine transport by the mixed membrane vesicles is linear for 10–20 min, whereas [U-^{14}C]inosine uptake is linear for approximately 2–5 min. The intravesicular product of [8-^{14}C]inosine uptake by unwashed, mixed membrane vesicles is hypoxanthine; no inosine is found in the vesicles. (A) K_m determination for the uptake of total radioactivity and ribose-1-P from [U-^{14}C]inosine. Mixed vesicles were preincubated for 10 min at 37 °C and then exposed to inosine concentrations between 20 μM and 150 μM for 5 min. Values obtained from a control series to which radioactive label was added after dilution of the membranes were subtracted from the experimental series. Corrected experimental values were then plotted as a double-reciprocal plot of uptake rate vs. inosine concentration. The K_m for [U-^{14}C]inosine uptake (▲) extrapolates to 64.5 μM. The K_m for ribose-1-P (■) accumulation determined after elution from filters and thin-layer chromatography extrapolates to 33 μM. (B) K_m determination for the accumulation of total radioactivity and from [8-^{14}C]inosine. Total radioactivity: the experiment was conducted as described for A with the exceptions that [8-^{14}C]inosine was substrate and the incubation time was 18 min (▲). The K_m for hypoxanthine accumulation (●) determined after elution from filters and thin-layer chromatography extrapolates to 133 μM. The K_m for [8-^{14}C]inosine uptake extrapolates to 125 μM. Accumulation of hypoxanthine was followed to determine rate of accumulation as a function of inosine concentration.

At least two mechanisms can be further distinguished by use of [8-^{14}C]inosine which only monitors the hypoxanthine accumulation, reflecting uptake of intact inosine alone and by use of different inhibitors. Formycin B inhibits uptake of [U-^{14}C]inosine by 50% at 0.8 mM, but does not inhibit further at higher concentrations, suggesting it may not affect the intact inosine mechanism. The data in Figure 4 show the differential effect of adenine on uptake of radioactivity from uniformly and specifically labeled inosine. Finally, *N*-ethylmaleimide also differentially inhibits the two systems with 50% inhibition for [8-^{14}C]inosine uptake (intact inosine mechanism) at 1.2 mM

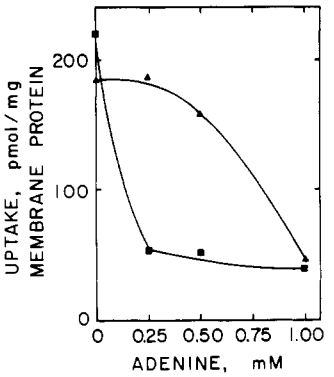

FIGURE 4: Inhibition of inosine uptake by adenine. Two reaction series containing various adenine concentrations were prepared and treated identically, with the exception that [U-^{14}C]inosine was added to one series (△) and [8-^{14}C]inosine was added to the other (▲). Incubation time for the reaction series [U-^{14}C]inosine was 5 min, and for the series [8-^{14}C]-inosine was 18 min. Reactions were terminated and total accumulation of radioactivity was determined.

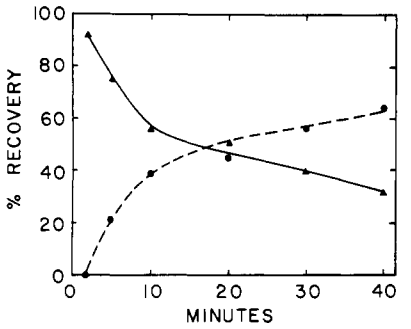

FIGURE 5: Intravesicular products from the use of adenosine as an uptake substrate. The transport reaction was carried out as described in Figure 2, using [8-^{14}C]adenosine. Filters were eluted, lyophilized, and chromatographed. The only labeled products detected were inosine (▲) and hypoxanthine (●). The data are plotted as percent recovery of each relative to total intravesicular radioactivity.

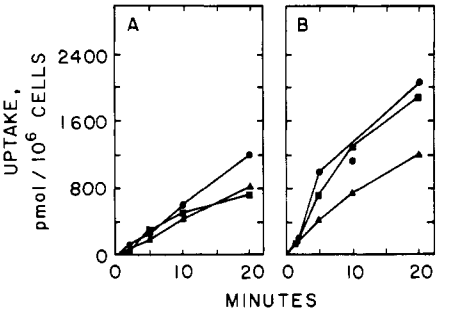

FIGURE 6: Uptake of hypoxanthine and inosine into intact BHK cells. Cells were grown to near confluence in 720-cm^2 roller bottles using either BHK medium (panel A) or BHK-HAT medium (panel B) containing hypoxanthine (2×10^{-5} M), amethopterin (3×10^{-6} M), and thymidine (2×10^{-5} M) (panel B). The cells were harvested, resuspended at about 10^6 cells/mL, and distributed in 0.1-mL aliquots in BHK medium buffered with 5 mM Hepes, pH 7.4. Samples were preincubated at 37 °C for 10 min and exposed to [8-^{14}C]hypoxanthine (90 μM, ●), [8-^{14}C]inosine (90 μM, ■), or [U-^{14}C]inosine (90 μM, ▲). Reactions were terminated at the times indicated by washing twice with 1 mL of warm BHK and rapid filtration onto nitrocellulose filters (0.45 μm). The filters were dried and monitored for content of radioactivity as described for vesicle uptake assays.

while 50% inhibition for [U-^{14}C]inosine uptake required 0.4 mM.

Unfortunately, we did not find an inhibitor or circumstance in which we could monitor activity of the phosphorylase-dependent step (leading to the accumulation only of ribose-1-P while the intact inosine mechanism was abrogated). Nevertheless, two mechanisms are indicated from the kinetic data and all other combinations of mechanisms are ruled out for the following reasons. (1) Any mechanism involving uptake of hypoxanthine or ribose-1-P was not found. Uptake of intact inosine must occur, therefore, to account for the hypoxanthine accumulation. (2) A second mechanism is required—leading to greater initial accumulation of ribose-1-P, but in which ribose-1-P is not the substrate. Thus, this second mechanism must be the phosphorylase-mediated translocation of the ribose moiety of inosine. Both mechanisms may go on simultaneously on the same vesicles or on different vesicles.

Metabolism and Transport of Adenosine by the Mixed Membrane Vesicle Population. Uptake of radioactivity from [U-^{14}C]adenosine and [8-^{14}C]adenosine occurs in a manner similar to inosine (data omitted). When adenosine is substrate, the intravesicular products are inosine, hypoxanthine, and ribose-1-P. Inosine appears in the vesicles when adenosine is substrate but not when inosine is substrate; no adenosine is seen in the vesicles. Deamination could occur before uptake, but then the inosine would be indistinguishable from the situation in which it serves as substrate. It could occur during or after uptake and be rapid enough to exclude observation of intravesicular adenosine. However, if occurring at this rate in vesicles capable of equally rapid inosine phosphorolysis, it would likewise result in the appearance only of hypoxanthine and ribose-1-P. Finally, there is a precursor-product relationship in the appearance of hypoxanthine from inosine when vesicles have been exposed to [8-^{14}C]adenosine (Figure 5).

Thus, the appearance of inosine in vesicles exposed to adenosine suggests that these vesicles have much lower phosphorylase activity or content and thus either adenosine inhibits the phosphorylase or distinct vesicles take up adenosine and inosine. These results indicate that much of the adenosine is initially taken up as an intact nucleoside, either in aminated or deaminated form. If the deaminase and the phosphorylase interacted, this could provide another explanation for possibly the same vesicles distinguishing adenosine-derived inosine from ordinary inosine (cf. Hochstadt-Ozer, 1972, for a similar situation involving this phosphorylase in bacteria).

Uptake by Intact Cells in Relation to Vesicle Uptake. Cells were grown on BHK medium or on BHK-HAT (hypoxanthine, 2×10^{-5} M, amethopterin, 3×10^{-6} M, thymidine, 2×10^{-5} M) medium. Under these conditions, the cells must use the exogenous base as purine precursor. Figure 6 shows the rate of uptake of [8-^{14}C]inosine, [8-^{14}C]hypoxanthine, and [U-^{14}C]inosine after growth on BHK or BHK-HAT. The cells grown on BHK-HAT take up [8-^{14}C]hypoxanthine and [8-^{14}C]inosine at double the normal rate. Uptake of [U-^{14}C]-inosine appears also at an increased rate, but not quite as increased as for the base or base labeled nucleoside. These data suggest that, in intact BHK cells, as well as in isolated vesicles, more than a single mode of inosine uptake appears to occur and that uptake appears to be inducible. If comparisons are made between uptake in intact cells and in isolated vesicles, the *metabolizing* intact cells take up *and incorporate* these compounds 20–50 times faster. However, if the cells are metabolically poisoned so that metabolism in the cells is as limited by virtue of the inhibitor as it is by virtue of purification in the vesicles, then the rates of the two are identical (cf. Plagemann and Erbe, 1972, for rates of uptake in nonmetabolizing cells).

Transport and Enzyme Activity in Purified Plasma Membrane Vesicles. Membrane vesicles were fractionated as described in Experimental Procedure in order to separate

TABLE II: Uptake of Inosine by Purified Plasma Membrane Vesicles; Total Counts per Minute and Percent Distribution of Products.

Substrate	Incubation (min)	Ribose-1-P cpm	Ribose-1-P %[a]	Inosine cpm	Inosine %[a]	Hypoxanthine cpm	Hypoxanthine %[a]
[U-^{14}C]Inosine	5	335	58.2	150	26	90	15.6
	10	630	53.2	460	38.8	95	8.0
	19.5	1000	71.5	320	22.8	80	5.7
[8-^{14}C]Inosine	10[b]			920	90.1	100	9.8
	20			1110	83.4	220	16.5
	30			1530	87.9	210	12.0

[a] % of total radioactivity in vesicles. [b] Longer incubation times were used because of the lower initial rate of the reaction leading to the accumulation of the purine portion of the molecule.

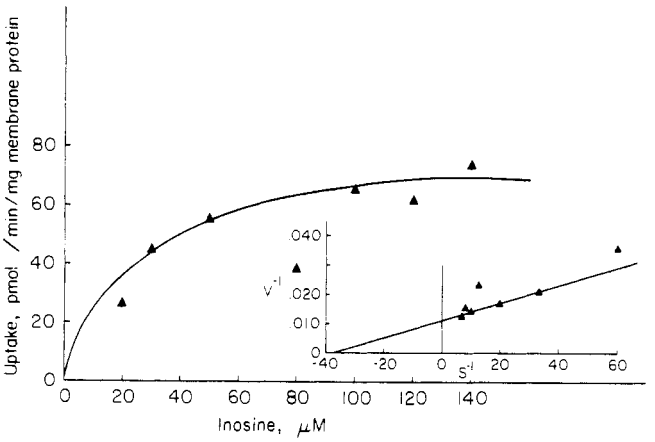

FIGURE 7: Uptake of [U-^{14}C]inosine by purified plasma membrane vesicles. Vesicles were purified and assayed as described under Experimental Procedure. Fifty micrograms of plasma membrane protein was used for each reaction. The reaction was carried out for 5 min. Results are depicted as a function of inosine concentration and also (in inset) as the double reciprocal of inosine concentration and uptake. The V_{max} for uptake extrapolates to 90 pmol min^{-1} mg^{-1} membrane protein; the K_m extrapolates to 28 μM.

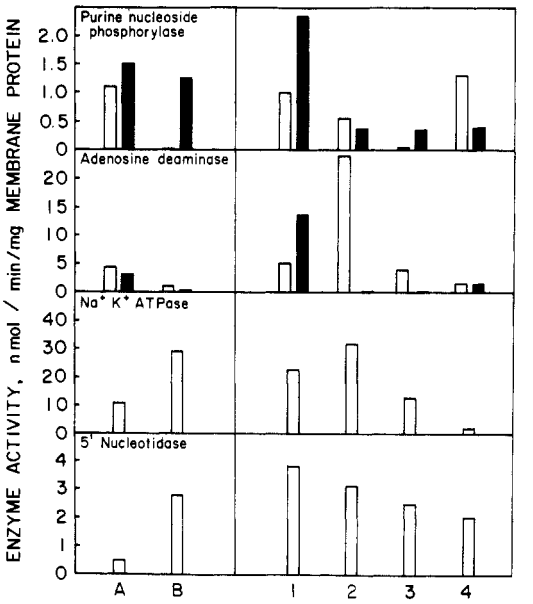

FIGURE 8: Distribution of various enzyme activities in subfractions of the plasma membrane. Four enzyme activities were followed before and after the final stages in plasma membrane purification. The left half of the left panels (A) shows enzyme activity in the mixed vesicles without Triton (open bar) and in the presence of 0.06% Triton (closed bar). The right half of the left panel (B) shows the enzyme activity of the washed, transiently lysed mixed vesicle population with and without 0.06% Triton. Where no open bar is shown, negligible activity was observed unless Triton was present (e.g., purine nucleoside phosphorylase in the washed mixed vesicles). Where no closed bar is shown, the effect of Triton was not tested (e.g., (Na^+, K^+)ATPase and 5'-nucleotidase). The right panel shows the enzyme activities and the effect of Triton on four membrane fractions recovered from gradient separation on dextran 110. Band 1 has the lowest buoyant density, while bands 2, 3, and 4 are recovered banding at increasing buoyant densities. Enzyme assays are performed as described in Experimental Procedure after removal of the dextran by dilution in Tris-sucrose buffer and collection by centrifugation at 100 000g for 1 h in the cold.

plasma membrane vesicles from endoplasmic reticulum. Adenosine transport activity did not survive the fractionation, whereas the highest inosine uptake activity was found in that fractionation with the lowest bouyant density (cf. Figure 1). Kinetics for inosine uptake in this fraction are shown in Figure 7. The K_m value (28 μM) for uptake of [U-^{14}C]inosine by the purified plasma membrane subfraction is less than half the value measured for the mixed membrane vesicle population, but is approximately the same as that of ribose-1-P accumulation in mixed vesicles. This is not unexpected since the uptake of [U-^{14}C]inosine reflects a composite of both mechanisms and would change as the ratio of one to another might change. The measurement of [8-^{14}C]inosine uptake, however, measures only the uptake of intact inosine. This shift in ratio of one mechanism vs. another would be seen in accumulation products (Table II) and in K_m values for [U-^{14}C]inosine and poses the possibility that both mechanisms may not reside on the same vesicles or come from the same cell. Table II and Figure 2 also show that, while the phosphorylase-dependent mechanism leading to the accumulation of ribose-1-P is relatively enriched in these vesicles compared with the less purified ones, the vesicles carrying out uptake of intact inosine appear to have very little phosphorylase activity capable of utilizing internal inosine as substrate (cf. Li and Hochstadt, 1976b; Hochstadt and Quinlan, 1976). Thus, the internal inosine breaks down more slowly. However, it is also possible that the loss of nonmembrane associated phosphorylase during the hypotonic washing step in the membrane purification could account for the slow-down in internal inosine breakdown. Figure 8 also shows significant enrichment in total phosphorylase occurs between the washed mixed vesicles and plasma membrane steps.

Overall, the least dense fraction was the only one to consistently show significant transport activity and, therefore, only it was kinetically characterized (Figure 7 and Table II). This fraction showed approximately a threefold specific activity increase for the intact inosine uptake mechanism. Enrichment may be typically sixfold or higher for the phosphorylase-dependent ribose moiety uptake and for phosphorylase enzyme activity in the purified plasma membrane fraction compared with the mixed vesicles.

Table II shows product-distribution data from the uptake of inosine by purified plasma membrane vesicles. The data

indicate that inosine phosphorolysis is not substantial; the percent of [8-^{14}C]inosine remaining intact after uptake is relatively constant, even though the total cpm accumulated increases over time. When [U-^{14}C]inosine is the substrate, more than 50% of the accumulated product is ribose-1-P; yet hypoxanthine accumulation is from 5 to 15%. No IMP was ever detected in the chromatographed samples. At the same time that ribose-1-P is accumulating, inosine is not being substantially degraded, as was already indicated. Therefore, two mechanisms for uptake of radioactivity from inosine must be operating, one which accumulates ribose-1-P, and one which takes up inosine intact, as was discussed for mixed membrane vesicles.

All gradient fractions were assayed for enzyme activity. Four plasma membrane-associated enzyme activities were assayed relative to their distribution throughout the plasma membrane purification (Figure 8). Two enzymes, purine nucleoside phosphorylase and adenosine deaminase, have both been assayed in the presence and absence of 0.06% Triton. The effect of 0.06% Triton on (Na^+, K^+)ATPase and 5′-nucleotidase, two plasma membrane marker enzymes, was not tested. Differences in enzymes activity with and without Triton may be attributable to lipid content, sealed or fragmented state of the vesicles, or other factors. Moreover, the membrane appears to have been subfractionated, especially with respect to localization of adenosine deaminase.[2]

Discussion

In this paper we report on the ability of membrane vesicles from BHK cells to transport inosine. The data presented are difficult to reconcile with a single mechanism of inosine uptake. They further suggest that inosine and adenosine may not be taken up by the same individual vesicles.

After exposure to inosine, both ribose-1-P and hypoxanthine accumulate within the vesicles, but their appearance occurs at different initial rates and with different K_ms. Differing K_ms would tend to rule out differential leakage after a single uptake mechanism. Yet, neither ribose-1-P nor hypoxanthine are themselves substrates for uptake by these vesicles. Thus, the mechanisms which must be responsible are: (1) a mechanism which calls for the uptake and metabolism of intact inosine; and (2) a mechanism which leads to the preferential uptake of ribose-1-P. The second mechanism could involve membrane-associated purine nucleoside phosphorylase, which interacts with intact inosine and P_i, releasing hypoxanthine on the outside and ribose-1-P on the interior of the membrane vesicle, as has already been described in detail for both cells (Li and Hochstadt, 1976b) and plasma membrane vesicles (Li and Hochstadt, 1976a; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976) from two cell lines of mouse fibroblasts. This group translocation mechanism, involving purine nucleoside phosphorylase, results only in the intravesicular accumulation of ribose-1-P.

Inorganic phosphate can serve as the phosphate donor and the energy driving the reaction appears to be derived from the splitting of the *N*-glycoside linkage. In this reaction, neither hypoxanthine nor inosine in significant amounts enters the vesicles, but free, extravesicular hypoxanthine is capable of reversing the reaction and causing the efflux of ribose-1-P. Preloaded, intravesicular inosine is not a substrate for the phosphorylase (Li and Hochstadt, 1976b; Hochstadt and Quinlan, 1976) and only inosine from the outside can lead to additional ribose-1-P appearing in the vesicles. Thus, a vectorial phosphorolysis reaction appears to be responsible. Through the use of mutant cell lines incapable of phosphoribosylating hypoxanthine, the membrane phosphorolysis route of uptake of the total ribose moiety of inosine was observed to be the major, if not sole, route of inosine handling in intact cells (Li and Hochstadt, 1976b).

In the above mouse studies there was always a concern, however, that hypoxanthine, an uncharged compound, might leak out while ribose-1-P, an anion, might be impermeant. This concern continued, despite our observations (Quinlan et al., 1976) that the preloaded leak rate, the exchange rate, or the uptake rate for hypoxanthine under the relevant experimental conditions was totally incompatible with any conclusion but one, that if hypoxanthine had been intravesicular we would have been able to detect it. In the present study we observed the BHK vesicles have an additional capacity, that of accumulating the hypoxanthine from inosine. Thus free hypoxanthine can be retained after uptake in mammalian vesicles. In order for hypoxanthine to accumulate in the BHK vesicles, a second mechanism involving uptake of hypoxanthine and/or intact inosine into the vesicles beyond that observed in the mouse systems must be envisioned. Inosine might be broken down prior to uptake and hypoxanthine and ribose-1-P each might serve as transport substrates. However, in numerous attempts, we failed to demonstrate hypoxanthine uptake into these BHK membrane vesicles (even in the presence of P-ribose-PP). Nor could we demonstrate that exogenous, unlabeled ribose-1-P would inhibit labeled ribose-1-P accumulation. Thus, uptake after cleavage appears to be ruled out. Since the K_m values for hypoxanthine and ribose-1-P accumulation differ by fourfold and since the initial rates of accumulation also differ (Figure 2), a mechanism involving intact inosine uptake followed by intravesicular breakdown would also be inconsistent. This suggests that the mechanism of the ribose accumulation might be similar to what we had observed with mouse fibroblast cell-derived plasma membrane vesicles (Li and Hochstadt, 1976b; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976), involving the uptake of the ribose moiety of inosine via group translocation, mediated by membrane-associated purine nucleoside phosphorylase. This would leave hypoxanthine entirely on the outside of the vesicles, such that a second mechanism would be required to account for hypoxanthine accumulation. Since free hypoxanthine was not a substrate for uptake into these membrane vesicles, the second mechanism must involve the uptake of intact inosine followed by its intravesicular breakdown to ribose-1-P and hypoxanthine.

If these are the two mechanisms operating, then the K_m for hypoxanthine accumulation (133 μM) should reflect the K_m for uptake of the intact inosine mechanism. It would also be the K_m for total uptake when [8-^{14}C]inosine (125 μM, Figure 3) is substrate. This is precisely what we found: the K_m was between 125 and 135 μM for all these measurements. The K_m for accumulation of ribose-1-P, however, would have two components, one equal to the hypoxanthine-accumulation K_m, reflecting the contribution of ribose-1-P from the intact inosine uptake mechanism, and another component, reflecting the phosphorylase-dependent translocation (cf. Li and Hochstadt, 1976a,b; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976). If the two rates or K_m values were reasonably close, it might be difficult to discern bimodality in double-reciprocal curves.

[2] It should be noted that these enzymes cannot be claimed to be adsorbed or trapped since they are not removed by the low ionic strength washes and transient lysis to allow the release of materials trapped during vesiculation. The true membrane localization in situ of these enzymes has been well established by these means for other cell lines (Li and Hochstadt 1976; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976).

Though we completely failed to demonstrate hypoxanthine uptake in BHK vesicles (with or without added PRPP), intact cells can use hypoxanthine (Figure 6); the hypoxanthine transport system in BHK cells, therefore, appears to be inactivated or lost during vesicle preparation. Thus, intact inosine must also be taken up and subsequently metabolized by either the same or a different purine nucleoside phosphorylase. This results in the accumulation of both hypoxanthine and ribose-1-PO_4.

These present results point up some notable comparisons to be made with our previous data. As already mentioned, it appears that hypoxanthine and inosine, which are uncharged molecules, do not leak out of the vesicles any faster or slower than does ribose-1-P, an anion. Our failure to have observed hypoxanthine or inosine in vesicles from mouse cells, therefore, further supports our previous contention, based on numerous other lines of evidence (Li and Hochstadt, 1976a,b; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976), that these compounds were never in the vesicles.

Inosine phosphorylase given its appearance on these membranes and on several other cell lines may be a useful plasma membrane marker (cf. Li and Hochstadt, 1976; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976) detectable on the BHK membranes. Though 8% of the purine nucleoside phosphorylase activity is recovered on the final plasma membrane preparation before treatment with Triton, this is a vast underestimation since many-fold activation of this enzyme occurs upon solubilization from the membranes (Figure 8). Enzyme activation with Triton treatment of the washed lysed vesicles to release trapped material shows similar activation in 3T3 cells (Hochstadt and Quinlan, 1976).

The data shown in Figure 8 caution the use of marker enzymes in bookkeeping unless detergent-treated and untreated samples are compared. We have previously discussed (Quinlan and Hochstadt, 1976; Jackman and Hochstadt, 1976; Hochstadt and Quinlan, 1976) the activity changes that membrane enzymes may undergo when mechanically solubilized or when treated with detergents.

In addition, there is evidence that inosine and adenosine may not be transported by the same vesicles even though we were unable to physically separate out vesicles having separate activities. In the mixed vesicle population, adenosine uptake results in the intravesicular accumulation of inosine, ribose-1-P, and hypoxanthine. In the same vesicle population, when inosine is substrate, no free inosine is ever observed in the vesicles, being immediately degraded to hypoxanthine and ribose-1-P. Thus, either multistep, allosteric interactions occur involving adenosine or adenosine deaminase (or both) controlling the activity of purine nucleoside phosphorylase, or uptake of adenosine occurs by vesicles which have less phosphorylase (or less active enzyme) than the vesicles that take up intact inosine. In both cases deamination of adenosine occurs in or on the vesicles as we have previously observed for L-cell and Balb/c 3T3 membrane vesicles (Li and Hochstadt, 1976b; Quinlan and Hochstadt, 1974). In intact L cells (Li and Hochstadt, 1976b), however, it has been our experience that the uptake of adenosine does not predominantly involve deamination. It has also been observed that adenosine is deaminated at the surface of the cell in Novikoff hepatoma cells, with inosine accumulating in the medium (Plagemann, 1971) and in rabbit polymorphonuclear leukocytes (Taube and Berlin, 1972) at a nonspecific site extremely early in its utilization.

Furthermore, the intravesicular inosine taken up by the purified plasma membrane vesicles, as well as inosine originating from adenosine (in mixed vesicles), is substrate for phosphorolysis inside the vesicle. This is clearly in contrast to our finding for L cell vesicles in which no mechanism for uptake of intact inosine was observed and in which inosine preloaded into the L-cell vesicles was not a substrate for the phosphorylase (Li and Hochstadt, 1976b; Hochstadt and Quinlan, 1976). These distinct results with the mouse membrane vesicles (Li and Hochstadt, 1976a,b; Quinlan and Hochstadt, 1976; Quinlan et al., 1976; Hochstadt and Quinlan, 1976) and, as presented here, with the membrane vesicles from the BHK line derived from the Syrian hamster suggest there exists a variety of transport mechanisms available to mammalian cells for the uptake of nucleosides. Moreover, more than one mechanism may exist in a single clonal cell population at one time. These mechanisms may alternate during the BHK cell cycle and, thus, might not be found simultaneously on the same BHK cell or vesicle.

Finally, we have shown that in BHK cells the uptake of hypoxanthine and inosine appears to be capable of being induced or stimulated by growth on BHK-HAT medium.

Note Added in Proof

A preliminary communication by Cohen and Amos ((1977), *J. Biol. Chem. 252,* 4428-4430) appeared after this paper was accepted which suggests, based on uptake studies with human cells, that the same two mechanisms we have identified and analyzed in detail here may operate in human cells as well.

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