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THE EXISTENCE OF A GROUP TRANSLOCATION TRANSPORT MECHANISM IN ANIMAL CELLS: Uptake of the Ribose Moiety of Inosine

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After exposure to inosine, transport-competent plasma membrane vesicles isolated from SV -40-transformed Balb/c 3T3 cells accumulate intravesicular ribose 1-PO_4 at a concentration 200-fold greater than the extravesicular concentration. An analysis of the purine nucleoside phosphorylase activity distribution in various subcellular fractions, relative to other enzyme activities, indicated the presence of plasma membrane-associated purine nucleoside phosphorylase activity. The plasma membrane vesicles appear relatively impermeable to hypoxanthine. However, hypoxanthine, which is a competitive inhibitor of the transport reaction, is the only compound tested capable of mediating efflux of already accumulated ribose 1-PO_4. In addition, hypoxanthine does not result in the efflux of transported uridine which is accumulated in these membrane vesicles as uridine. Exogenous ribose 1-PO_4 neither results in counterflow nor does it inhibit the original uptake reaction. The following transport reaction is proposed: uptake occurs by group translocation, mediated by membrane-localized purine nuceloside phosphorylase. The data are consistent with sites for inosine and hypoxanthine being on the outer membrane surface whereas the ribose 1-PO_4 site is only on the inner surface.

INTRODUCTION

The mammalian cell membrane has recently become the focus of considerable investigation into such diverse problems as cell adhesion (1), hormone action (2–4), and growth control phenomena (5–7). Cell-free membrane preparations, in which membrane components have been separated from other cell constituents, have often served as the experimental systems for such investigations (7–11). A major question which often arises is whether component relationships in the isolated membrane are reflective of in situ orientations and functional interactions. We believe that the study of membrane transport systems may serve as an extremely sensitive tool with which to examine overall membrane integrity and membrane component interrelationships.

Transport is a highly specific membrane function which requires an intact membrane in order to proceed, whereas most enzymatic or binding functions carried out by constituent membrane proteins may be able to, and often do, proceed after membrane dissolution. Furthermore, certain types of transport systems may be especially useful. For example, group translocation (12), in which the transport substrate is covalently altered

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during its passage across the membrane, may be particularly suited for study since a highly specific orientation of the membrane transport "carrier"-enzyme is required in order to effect, simultaneously, transport and catalysis. For those group translocation systems which have been identified, such an alteration in the orientation of the "carrier"-enzyme in the membrane would mean vectorial phosphorolysis (13) or vectorial phosphorylation (12) being converted to a nonvectorial process in which metabolism proceeds on the outer membrane surface but all the products are released to that same surface. That is, all the products are released on the outside of the cell or vesicle. With isolated bacterial membranes (12) it was observed that NaF could influence the PEP-sugar phosphotransferase system so as to effect release of the glucoside-P product on either the inner or the outer membrane surface. More recently, the specificity of membrane enzymes has been shown to be more restricted on the membrane than when they are released from the membrane into aqueous solution (14). Thus, though the two other prevalent transport mechanisms (facilitated diffusion and "classical" active transport) may be involved in growth control, group translocation represents the only transport process in which unreacted substrate can be distinguished from molecules which have interacted with the transport-catalytic site but are either not transported or have "leaked" out. Group translocation, then, can serve as an extremely sensitive means of studying membrane component orientation and function.

A relationship between transport and cell growth control phenomena has been observed for uridine transport in animal cells (5) and their derived membranes (7), and, by use of bacterial membranes (15), for purine base uptake as related to amino acid control of macromolecular biosynthesis. These observations further support the concept (6) that membrane transport, and membrane systems in general, may play a key role in cellular regulatory processes and in human disease states. Therefore, studies into the fundamental mechanisms of substrate transport across animal cell membranes should continue to be a productive field of research. The use of isolated membrane vesicles and possible or putative group translocation systems appears to provide very useful experimental tools with which to approach studies of cell growth control and disease states.

The report which follows details the study of inosine interaction with isolated plasma membrane vesicles from SV-40-transformed Balb/c 3T3 cells. This interaction results in the intravesicular accumulation of ribose 1-PO_4 and the extravesicular accumulation of hypoxanthine. This transmembrane catalysis or vectorial phosphorolysis of a nucleoside across an animal cell membrane represents the first demonstration of a group translocation transport process in higher organisms similar to the translocation of certain monosaccharides (12, 16) and bases and nucleosides (17–20) across membranes of enteric bacteria, and the translocation of lactose in Staphylococcus aureus (21).

ISOLATION OF PLASMA MEMBRANE VESICLES

We have used the simian virus 40-transformed Balb/c mouse 3T3 (SV-3T3) cell line for these studies. Growth conditions have been described previously (7). Confluent cultures are harvested by scraping and the cells are disrupted by nitrogen cavitation. Transport-competent plasma membrane vesicles are isolated by the use of differential

centrifugation and a discontinuous dextran gradient. One of the final steps in the isolation procedure involves the treatment of a mixed vesicle population (containing exclusively plasma membrane and endoplasmic reticulum) with a series of wash steps which remove adsorbed protein and protein trapped within the vesicles. This washed mixed vesicle population can then be separated into plasma membrane and endoplasmic reticulum components by use of a dextran gradient centrifugation step. Details of cell harvesting, disruption, and fractionation have been described previously (9, 13). The final yield of plasma membrane material is 30–40%, based on 5′-nucleotidase activity (9, 13). The plasma membrane material is contaminated with mitochondria (based on succinate dehydrogenase activity) to the extent of 2.5%, with endoplasmic reticulum (based on NADH dehydrogenase activity) to the extent of 3%, and with lysosomal material (based on p-nitrophenylsulfatase activity) to the extent of $< 1\%$ (9, 13). Thus, the plasma membrane vesicles used for transport analysis are highly purified and of a relatively high yield.

USE OF INOSINE AS A TRANSPORT SUBSTRATE

As shown by the data in Fig. 1, the use of ^{14}C-U-inosine as a transport substrate results almost exclusively in the intravesicular accumulation of ribose 1-PO_4; only trace amounts of inosine and hypoxanthine are found. The total accumulation of radioactivity is linear for 5–10 min, after which a plateau is observed. The apparent K_m for inosine, based on the accumulation of ribose 1-PO_4, is 35–45 μM (13).

Under the experimental conditions used, the combined intra- and extravesicular products of inosine metabolism are hypoxanthine and ribose 1-PO_4. In addition, this conversion has been found to be phosphate dependent (13). Therefore, the involvement of purine nucleoside phosphorylase (E.C. 2.4.2.1.) was investigated. This enzyme catalyzes the following reaction:

purine nucleoside + phosphate $\rightleftharpoons$ purine base + ribose 1-PO_4

We have observed different results, depending on the cell source from which the plasma membrane vesicles were isolated. That is, SV-3T3 cells which are allowed to become highly confluent ($4–6 \times 10^5$ cells/cm^2) prior to harvesting yield plasma membrane vesicles which accumulate almost exclusively ribose 1-PO_4 from inosine (the ribose 1-PO_4 to hypoxanthine ratio is 15:1; see Fig. 1). Plasma membrane vesicles derived from monolayer cultures ($2–4 \times 10^4$ cells/cm^2) of SV-3T3 or 3T3 cells accumulate both ribose 1-PO_4 and hypoxanthine (at a ratio of 3:1, respectively) from inosine. However, monolayer culture-derived plasma membrane vesicles accumulate only the phosphorylated pentose sugar if unlabeled ribose 1-PO_4 is present during the transport assay. Ribose 1-PO_4 does not inhibit uptake with either inosine or hypoxanthine as transport substrate. In addition, ribose 1-PO_4 itself is not transported. Thus, we believe that exogenous ribose 1-PO_4 affects the intravesicular accumulation of hypoxanthine from inosine by preventing, in a product-inhibition manner, the production of hypoxanthine from inosine by purine nucleoside phosphorylase located on membrane sheets or on "leaky" vesicles. That is, the hypoxanthine which is found intravesicularly when inosine is used as a transport substrate probably comes from the production of hypoxanthine by purine nucleoside phosphorylase and the subsequent transport of the purine base into the membrane vesicles.

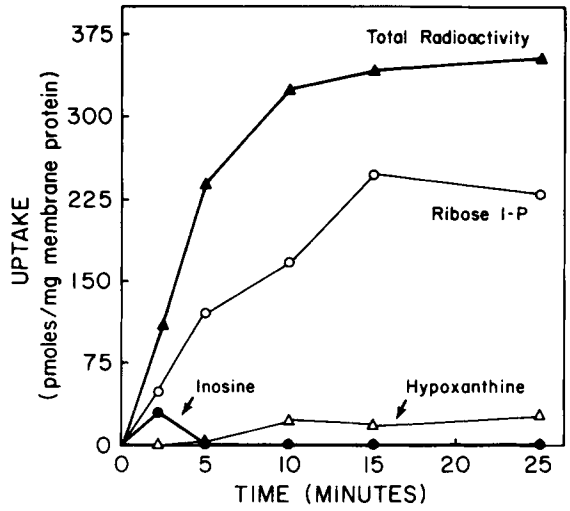

Fig. 1. Total accumulation of radioactivity by SV-3T3 cell-derived plasma membrane vesicles and chromatographic analysis of the transport products. The transport assay reaction mixture contains, in a total volume of 50 μl: 75 μg membrane vesicle protein, 50 mM potassium phosphate buffer (pH 7.6), and 100 mM sucrose. The reaction is initiated at 37°C with 60 μM ^{14}C-U-inosine (260 mCi/mmole). At various times thereafter, the reaction is terminated with 20 volumes of warm 0.8 M NaCl and membranes are collected on 0.3 μm nitrocellulose filters. These filters can be dried and monitored for total content of radioactivity, or the filter can be eluted with water and the eluate lyophilized, resuspended, and spotted on thin layer chromatography sheets which are developed in an appropriate solvent. Chromatographically separated transport products can then be monitored for content of radioactivity. Additional experimental details have been described previously (7–9, 13).

POSSIBLE TRANSPORT MECHANISMS

Plasma membrane vesicles from confluent SV-3T3 cells, when exposed to inosine as a transport substrate, accumulate ribose 1-PO_4 and hypoxanthine at a ratio of 15:1, respectively. Figure 2 is a schematic diagram of three mechanisms by which such a product distribution could occur. Panel A represents a model in which the phosphorolysis takes place prior to transport, such that ribose 1-PO_4 itself serves as the actual transport substrate. This mechanism implies that the accumulation of ribose 1-PO_4 from inosine by these plasma membrane vesicles should be inhibitable by the inclusion of unlabeled ribose 1-PO_4 in the transport reaction mixture. Also, it should be possible, at steady state, to induce a counterflow reaction by use of exogenous, unlabeled ribose 1-PO_4.

Panel B of Fig. 2 represents a mechanism in which phosphorolysis is carried out in a single step, referred to as a group translocation event. Group translocation systems have been described in procaryotic cells (16, 17, 22), but not in eucaryotic cells. The existence of a group translocation reaction which mediates the intravesicular accumulation of ribose 1-PO_4 from inosine via plasma membrane-associated purine nucloside phosphorylase

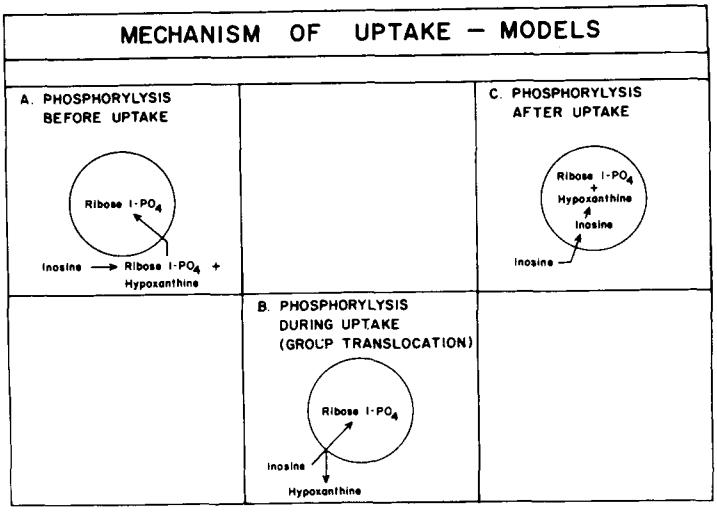

Fig. 2. Various models are proposed which demonstrate how the ribose moiety of inosine can be accumulated as ribose 1-PO_4 by plasma membrane vesicles. If phosphorolysis occurs before uptake (panel A), then ribose 1-PO_4 should inhibit the transport reaction. If phosphorolysis occurs as a group translocation event, then hypoxanthine might reverse the transport reaction, leading to the efflux of already accumulated ribose 1-PO_4. If phosphorolysis occurs after inosine itself is transported, then hypoxanthine should only induce ribose 1-PO_4 efflux at a rate consistent with the rate of which hypoxanthine enters the vesicles.

might have the following properties. Since transport and metabolic conversion would be carried out by the same molecule (purine nucleoside phosphorylase), effector sensitivities and kinetic constants might be very similar when transport activity (intravesicular accumulation of ribose 1-PO_4 during transport) and phosphorylase activity (conversion of inosine to hypoxanthine and ribose 1-PO_4 by the enzyme) are measured individually. Another characteristic of group translocation would be that the ribose moiety of exogenous inosine would be preferentially phosphorylated relative to ribose from intravesicular, preloaded inosine. Finally, it might be possible to reverse phosphorolysis (that is, reform inosine from ribose 1-PO_4 and hypoxanthine) using exogenous hypoxanthine. For example, if hypoxanthine were found to be a competitive inhibitor of the transport reaction, hypoxanthine might result in the efflux of ribose 1-PO_4 and its concomitant conversion to inosine as it passes through the membrane into the extravesicular fluid. Also, this induced efflux could occur at a much faster rate than could be explained by hypoxanthine permeation alone, since hypoxanthine would not have to enter the vesicle to cause the efflux of ribose 1-PO_4.

A third mechanism (panel C of Fig. 2) which we have postulated to explain the observed transport reaction involves posttransport phosphorolysis. That is, inosine is transported into the vesicle intact and then is acted upon by intravesicular purine nucleoside phosphorylase; the ribose 1-PO_4 remains inside the vesicle, whereas the hypoxanthine is transported or diffuses out of the vesicle. The existence of this mechanism could mean that the transport event and metabolic event, caused by two separate molecules,

would have different characteristics in terms of effector sensitivities and kinetic constants. While hypoxanthine could still induce the efflux of intravesicular ribose 1-PO_4, requiring the uptake of hypoxanthine followed by production of inosine and exit of the latter, the efflux rate would not be greater than the entrance rate for hypoxanthine. Finally, if the purine nucleoside phosphorylase enzyme were simply "trapped" in the vesicle as a result of the cell disruption procedure, then the final steps in plasma membrane purification which remove trapped protein should remove purine nucleoside phosphorylase activity to the same extent as other putative cytosol enzymes.

EXPERIMENTAL EVIDENCE CONSISTENT WITH GROUP TRANSLOCATION AND INCONSISTENT WITH PRE- OR POSTTRANSPORT PHOSPHOROLYSIS

One of the transport mechanisms postulated to account for the intravesicular accumulation of ribose 1-PO_4 from inosine involved pretransport phosphorolysis and the subsequent uptake of ribose 1-PO_4 by the plasma membrane vesicles. However, the data in Fig. 3 rule against this possibility since the inclusion of various concentrations of unlabeled ribose 1-PO_4 failed to inhibit the transport reaction. In addition, as shown by the data in Fig. 5, a counterflow phenomenon did not result upon the addition of exogenous, unlabeled ribose 1-PO_4 to plasma membrane vesicles which had accumulated, to a steady-state level, labeled ribose 1-PO_4 from labeled inosine. That is, if ribose 1-PO_4 itself had been the transport substrate, then the entry of the unlabeled ribose 1-PO_4 into the vesicles would have resulted in a concomitant efflux of internal, labeled ribose 1-PO_4. Thus, pretransport phosphorolysis does not seem to be the mechanism by which SV-3T3-derived plasma membrane vesicles accumulate the ribose moiety of inosine.

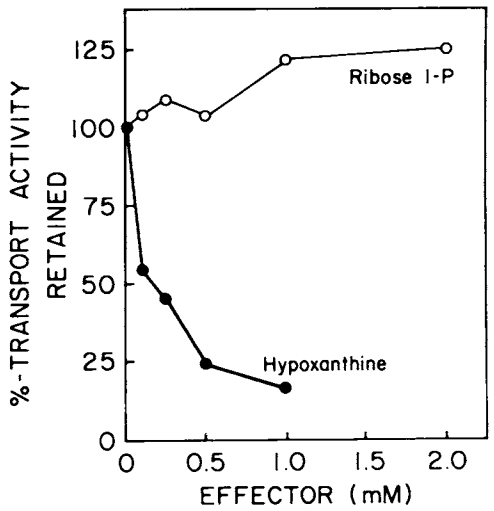

Fig. 3. Exogenous hypoxanthine, but not exogenous ribose 1-PO_4, inhibits the accumulation of ribose 1-PO_4 from inosine by plasma membrane vesicles. Transport activity at 0 and 3 min is measured in the presence of various concentrations of effector. The 100% transport activity value represents 176 pmoles ribose 1-PO_4 accumulated/3 min per mg membrane vesicle protein.

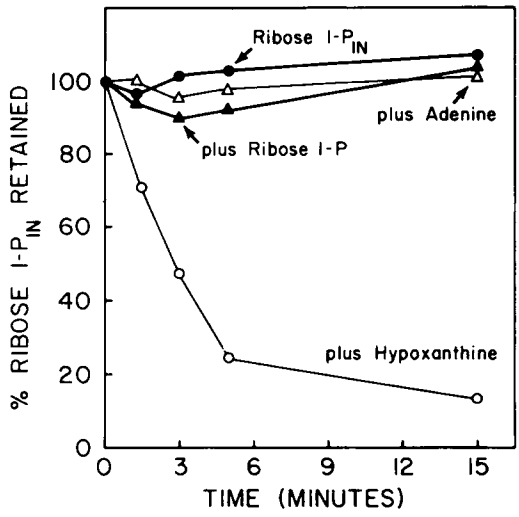

Fig. 4. Exogenous hypoxanthine, but not exogenous adenine of ribose 1-PO_4, induces the efflux of accumulated ribose 1-PO_4, labeled "ribose 1-P_{IN}." Plasma membrane vesicles accumulate ^{14}C-U-ribose 1-PO_4 from 15 μM ^{14}C-U-inosine to a steady-state level and then are diluted 10-fold in reaction medium alone (ribose 1-PO_{4IN}) or medium containing 100 μM hypoxanthine, adenine, or ribose 1-PO_4. Reaction medium contains 50 mM potassium phosphate buffer (pH 7.6) and 100 mM sucrose. At various times thereafter, membranes are collected and assayed for ribose 1-PO_4 accumulated. The 100% "ribose 1-P_{IN}" value represents 126 pmoles ribose 1-PO_4 accumulated per mg membrane vesicle protein.

One way of distinguishing between a single-step, group translocation mechanism (involving a single "carrier"-enzyme) and a two-step, uptake-conversion mechanism (involving a separate "carrier" and enzyme) would be to show that each individual event, transport and phosphorolysis, has a different effector sensitivity and kinetic constant. Such an analysis has been carried out by Plagemann and Roth (23) and Plagemann and Erbe (24). Their results, based on uridine uptake by Novikoff rat hepatoma cells, showed that uridine transport was not dependent on its conversion to uridylic acid, via uridine kinase; in addition, the transport and kinase reactions each demonstrated quite different K_m values.

Similarly, Paterson and Simpson (25) used various purine ribonucleoside analogs to investigate purine nucleoside transport and phosphorolysis in human erythrocytes. Their results indicated that the transport step was much more sensitive to inhibition by 6-methylthioinosine than was cell-free phosphorolysis of inosine. Paterson and Simpson (25), therefore, concluded that purine nucleoside transport and cleavage occurred as two separate steps. However, it is possible that a single enzyme which exists in both a cytosol and a membrane-associated state could demonstrate different inhibitor sensitivities depending on whether it was embedded in the membrane or free in solution. A similar situation has been described (17) for the adenine phosphoribosyltransferase system in E. coli in which the phosphoribosyltransferase enzyme, though readily released in soluble form, exists in situ in a membrane-associated state. In the case of purine nucleoside transport by eucaryotic cells, in order to demonstrate that transport and phosphorolysis are

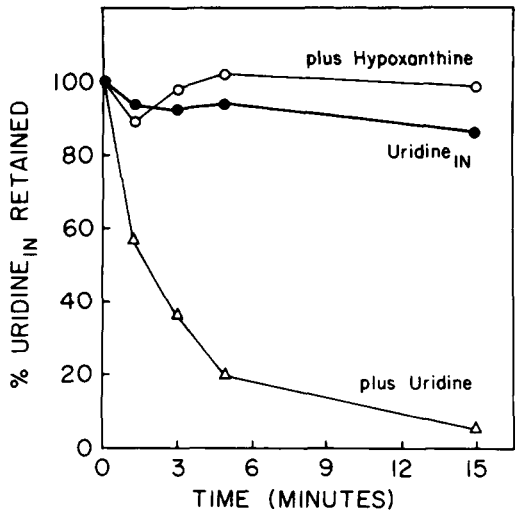

Fig. 5. Exogenous hypoxanthine does not induce the efflux of accumulated uridine, labeled "uridine$_{IN}$." Plasma membrane vesicles from SV-3T3 cells transport uridine without any significant metabolic conversion (7). Plasma membrane vesicles accumulate labeled uridine from 20 μM ^{14}C-U-uridine (500 mCi/mmole) to a steady-state level and then are diluted 10-fold in reaction medium alone (uridine$_{IN}$) or medium containing 500 μM hypoxanthine or uridine. "Reaction medium" is defined as in Fig. 4. At various times thereafter, membranes are collected and assayed for uridine accumulated. The 100% "uridine$_{IN}$" value represents 41 pmoles uridine accumulated per mg membrane vesicle protein.

separate events it would seem necessary to use an analog to which the cytosol enzyme is sensitive whereas transport is not, such that transport results in intravesicular accumulation of free nucleoside. Alternatively, one could search for an analog which is not phosphorolytically cleaved but is transported uncleaved into the cell or membrane vesicle by the same purine nucleoside "carrier" system.

Group translocation is defined as a process in which a membrane-associated enzyme carries out both a transport and metabolic step. This requirement, however, does not demand that this enzyme be tightly bound to the membrane, such that cell disruption would not dislodge it; in addition, the enzyme could exist simultaneously in a membrane-associated and cytosol state. We have investigated the interaction of purine nucleoside phosphorylase and the plasma membrane in terms of transport activity and enzyme activity. For example, as the data in Table I show, both transport activity (defined as the intravesicular accumulation of ribose 1-PO_4 from inosine) and purine nucleoside phosphorylase activity (defined as the total production of hypoxanthine and ribose 1-PO_4 from inosine in the presence of Triton X-100) copurify with the plasma membrane vesicles when the latter are separated out of the mixed vesicle population. Transport activity is enriched 4-fold in the plasma membrane vesicles relative to the mixed vesicle population; the enrichment in purine nucleoside phosphorylase activity is 5-fold. The endoplasmic reticulum contains only 10–20% of both transport and enzyme activity. Further evidence relating to the existence of plasma membrane-associated purine nucleoside phosphorylase

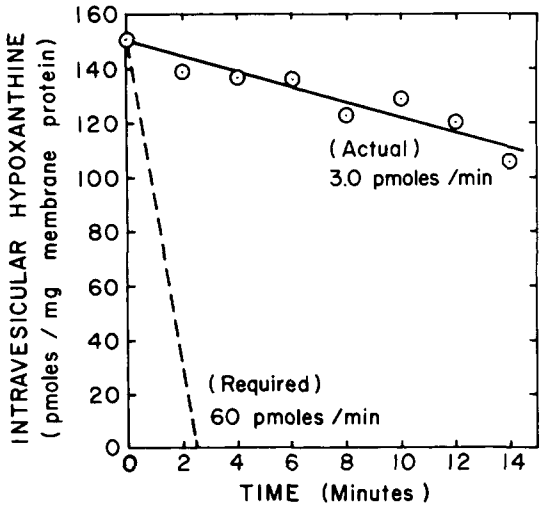

Fig. 6. Measurement of the rate at which hypoxanthine exits out of SV-3T3 cell-derived plasma membrane vesicles. Prewarmed (37° C) plasma membrane vesicles were osmotically shocked into 5-volumes of cold (2° C) 115 μM ^{3}H-G-hypoxanthine. Potassium phosphate and sucrose were then added to final concentrations of 50 mM and 100 mM, respectively (the intravesicular hypoxanthine concentration is 85 μM). The membrane reaction mixtures were then diluted 10-fold in reaction medium containing 100 μM unlabeled hypoxanthine. At various times thereafter, membrane vesicles were collected and assayed for content of labeled hypoxanthine (—○—). Also shown (-----) is the rate at which hypoxanthine would have to exit if inosine were taken up intact and degraded intravesicularly in order to account for the chromatography results.

TABLE I. Ribose 1-Phosphate Accumulation and Purine Nucleoside Phosphorylase Activity as a Function of Plasma Membrane Purification

Subcellular fraction	Transport activity	Phosphorylase activity
	pmoles ribose 1-PO_4 formed/3 min per mg membrane protein	
Unwashed mixed vesicles	49.2	1827.0
Plasma membrane	206.0	9384.0
Endoplasmic reticulum	39.6	594.0

Transport activity, with ^{14}C-U-inosine as substrate, was measured as described in Fig. 1. Purine nucleoside phosphorylase activity was assayed for in the same way except that a 3 μl aliquot of the reaction mixture was spotted directly, without nitrocellulose filter collection of membranes, on cellulose thin-layer chromatography sheets. Development and radioactivity monitoring was as described for Fig. 1. The purine nucleoside phosphorylase activity was measured in the presence of 0.02% Triton X-100. The mixed vesicle population contains both plasma membrane and endoplasmic reticulum membrane vesicles as described in the text.

activity is presented in Table II. The data represent an experiment in which the distribution of various enzyme activities has been measured as a function of plasma membrane purification. For example, uridine kinase and hypoxanthine phosphoribosyltransferase activities decrease markedly in the plasma membrane material relative to the mixed vesicle population from which the plasma membrane is isolated. However, both 5′-nucleotidase (a plasma membrane-marker in SV-3T3 cells; see references 9 and 13) and purine nucleoside phosphorylase activities are enriched 4- and 5-fold, respectively, in the plasma membrane vesicles as compared to the mixed vesicle population. Thus, despite only about 5–10% of the total purine nucleoside phosphorylase activity being plasma membrane bound following cell disruption and a quite extensive plasma membrane purification procedure, there does appear to be a specific association involved, especially when one follows the activity distribution of two enzymes believed to be exclusively cytosol-localized. The existence of membrane-localized enzyme that is predominantly recovered with the cytosol may be related to its hydrophilic nature. Conversely, the enzyme (one gene product or two) may actually exist on the membrane as well as in the cytosol in situ. That is, membrane-associated purine nucleoside phosphorylase could be involved in the transport and metabolism of extracellular purine nuceloside, whereas the cytosol-localized enzyme could function in the degradation of purine nucleosides which result from phosphatase action on intracellular nucleotides.

Perhaps the most interesting evidence we have obtained which is suggestive of a group translocation mechanism involves the use of hypoxanthine to induce the efflux of ribose 1-PO_4 out of the plasma membrane vesicles. As seen in Fig. 4, ribose 1-PO_4 which has accumulated intravesicularly from exogenous inosine can be caused to efflux in the presence of hypoxanthine, but not in the presence of adenine or ribose 1-PO_4. The rate at which ribose 1-PO_4 effluxes is proportional to the concentration of hypoxanthine added (13). Furthermore, as shown by the data in Fig. 5, the hypoxanthine effect is not due to the production of a "leak" since plasma membrane vesicle-accumulated uridine is not caused to efflux in the presence of hypoxanthine; SV-3T3 membrane vesicles transport uridine such that only free uridine accumulates (7). The rate at which the accumulated ribose 1-PO_4 exits in the presence of 100 μM hypoxanthine (Fig. 4) is 20 pmoles/min per mg membrane vesicle protein. The hypoxanthine-induced exit of ribose 1-PO_4 from the plasma membrane vesicles could be explained by either of two mechanisms. In the first, hypoxanthine could enter the vesicles by transport and/or diffusion and interact with ribose 1-PO_4 and purine nucleoside phosphorylase to reform inosine which then exits from the vesicles. Alternatively, extravesicular hypoxanthine could interact directly with membrane-associated purine nucleoside phosphorylase which in turn interacts with intravesicular ribose 1-PO_4, so that the group translocation reaction is reversed.

In order to interpret the efflux data and also to measure the rate at which hypoxanthine would exit from the plasma membrane vesicles, it was necessary to determine flux rates of hypoxanthine across the membrane. As shown in Fig. 6, the exit rate of hypoxanthine was obtained by preloading vesicles with labeled hypoxanthine (the internal concentration was 118 μM ribose 1-PO_4 at steady state, following transport) and measuring its exit into reaction medium containing an equal concentration of unlabeled hypoxanthine. The latter prevented re-entry of labeled hypoxanthine and, therefore, gave an estimate of the true rate at which hypoxanthine exits. This exit rate was

TABLE II. Distribution of Various Enzyme Activities in Membrane Fractions from SV-3T3 Cells

Subcellular fraction	Uridine kinase activity	Phosphorylase activity	5'-nucelotidase activity	Phosphoribosyltransferase activity
	nmoles product formed/30 min per mg protein (as in Table I).			
Total homogenate	10.1	122.0	2.10	78.3
Unwashed mixed vesicles	1.80	32.4	9.28	49.2
Plasma membrane	0.62	163.0	36.5	11.6
Endoplasmic reticulum	0.32	19.9	7.71	7.4

Uridine kinase activity was measured as the production of uridylic acid from uridine and ATP; purine nucleoside phosphorylase activity was measured as the production of ribose 1-PO_4 and hypoxanthine from inosine; 5'-nucleotidase activity was measured as the production of adenosine and inosine from adenylic acid; hypoxanthine phosphoribosyltransferase activity was measured as the production of inosine 5' -monophosphate from hypoxanthine and phosphoribosylpyrophosphate. Specific details of the assays have been described previously (9, 13). All enzyme assays were performed in the presence of 0.02% Triton X-100. The total homogenate refers to that material obtained immediately after nitrogen cavitation-disruption of the cells and prior to any centrifugation steps.

determined to be about 3.0 pmoles/min per mg membrane protein, which is to be compared to an exit rate of about 60 pmoles/min, the rate at which hypoxanthine would have to exist if post-transport phosphorolysis occurred. That is, if inosine was taken up intact and then underwent phosphorolysis, the resultant hypoxanthine would have to leave the vesicles at a minimum rate of 60 pmoles/min in order to obtain a final, intravesicular ribose 1-PO_4 to hypoxanthine ratio of 15:1, respectively. Similarly, we also measured the rate at which 100 μM labeled hypoxanthine enters vesicles preloaded with unlabeled hypoxanthine (data not shown); this rate was about 1–2 pmoles/min. Inosine-derived ribose 1-PO_4 was induced to efflux from plasma membrane vesicles in the presence of 100 μM hypoxanthine at a rate of 20 pmoles/min (see Fig. 4). In other words, one cannot explain hypoxanthine-induced efflux of ribose 1-P by hypothesizing that hypoxanthine enters the vesicles and interacts with intravesicular purine nucleoside phosphorylase and ribose 1-PO_4. However, ribose 1-PO_4 efflux can be explained by hypothesizing that hypoxanthine interacts on the outside surface of the vesicle with a transport-catalytic site which spans the membrane, a concept which supports the involvement of a group translocation mechanism in the intravesicular accumulation of the ribose moiety of inosine as ribose 1-PO_4.

Our model proposes that binding sites for inosine and hypoxanthine exist on the outside surface of the plasma membrane vesicle, while the ribose 1-PO_4 site is located only on the inner side of the membrane vesicle. Furthermore, we propose that plasma membrane-associated purine nucleoside phosphorylase serves both as a transport "carrier" and as a catalytic agent, performing both functions in a single-step process.

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