Transport Mechanisms in Isolated Plasma Membranes

NUCLEOSIDE PROCESSING BY MEMBRANE VESICLES FROM MOUSE FIBROBLAST CELLS GROWN IN DEFINED MEDIUM

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Plasma membrane vesicles were isolated from a subline of L1210 mouse fibroblasts grown on defined medium in the absence of serum. These vesicles were not significantly contaminated by mitochondria or endoplasmic reticulum. The isolation procedure, a modification of that originally developed by McKeel and Jarett (McKeel, D. W., and Jarett, L. (1970) J. Cell Biol. 44, 417-432) employs mechanical homogenization in isotonic medium followed by differential centrifugation. The resultant plasma membrane vesicles take up radioactivity when exposed to uniformly labeled nucleosides. Two subfractions of the plasma membrane were isolated, distinguished by their differing activity of 5'-nucleotidase and (Na⁺,K⁺)-stimulated ATPase, two well known plasma membrane enzyme markers. Uptake of nucleoside radioactivity was extensively studied in one subfraction; it was linear with time and membrane concentration over ranges used for the studies. Apparent $K_m$ values for uptake of radioactivity from adenosine, inosine, and uridine were $7.1 \pm 3.5 \mu M$, $53 \pm 26 \mu M$, and $6.9 \pm 3.5 \mu M$, respectively. Uptake of radioactivity from all three nucleosides exhibits a broad pH optimum from pH 7 to pH 9, but falls off rapidly at lower pH. N-Ethylmaleimide was an effective inhibitor of uptake of radioactivity from all three nucleosides; uptake of radioactivity from uridine is more sensitive than uptake of radioactivity from the purine nucleosides. Adenosine inhibited uptake of radioactivity from inosine more than from uridine. Inosine inhibited the uptake of radioactivity from adenosine, but uridine did not. Caffeine and 6-methylaminopurine riboside (6-N-methyladenosine) differentially inhibit uptake of radioactivity from adenosine and inosine, and thus the vesicles apparently possess separate transport systems for uptake of radioactivity from purine nucleosides and from uridine.

The surfaces of actively proliferating cells differ chemically and structurally from the surfaces of cells in density-inhibited cultures (2-8). These surface membranes also differ functionally in that nutrient transport rates appear concomitantly altered (9-14). It has been suggested that both the chemical changes (2-8) and, even more specifically, the transport changes (13) may be involved in growth regulation in normal cells, and in its failure in tumor cells. Previous results from this laboratory (14), using isolated membrane vesicles, have demonstrated that transport differences between quiescent and actively growing cells do represent alterations of the membrane itself, and not in some process subsequent to, but which in turn alters, the transport step. Another transport study, using Balb 3T3 plasma membrane vesicles, showed that the ribose moiety of inosine is translocated across the membrane in a purine-nucleoside phosphorylase-dependent step (15, 16). In this present paper and the paper which immediately follows it (17), we offer evidence relating to the transport processes involved in the handling of a variety of nucleosides by membrane vesicles prepared from a mouse fibroblast cell line grown in serum-free defined medium. In this first paper, basic kinetics, inhibitor effects, and effects of pH and heat are presented without regard to identification of precise biochemical mechanisms. The results will also show that plasma membranes isolated by the present procedure (18) can be separated into two fractions with different properties, further suggesting surface mosaicism (19). The second paper (17) represents an investigation of metabolism concomitant to the uptake observed in the vesicles, the enzymes involved in this metabolism, their relation to the membrane, and the possible mechanism(s) of transport. Because the uptake product in vesicles from L-cells turned out to be only a portion of the nucleoside, rather than the entire molecule, we refer to uptake in both papers as "uptake of radioactivity from the nucleoside" rather than "nucleoside uptake."

EXPERIMENTAL PROCEDURE

Materials—Radioactive chemicals were purchased from Amersham/Searle, Chicago, Ill. The specific activities of the uniformly $^1^3^C$-labeled

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nucleosides are: adenosine, 585 mCi/mmol; inosine, 250 mCi/mmol; guanosine, 601 mCi/mmol; and uridine, 509 mCi/mmol. Ficoll was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade or standard commercial products of reagent grade.

Cell Culture—The L-cells (NCTC clone 929 mouse cells) used in these experiments were a gift from Dr. Paul Kitos (20), and were grown in roller bottles (900 cm²) containing 150 ml of Waymouth medium MD05/1 (Grand Island Biological Co., Grand Island, N. Y.) without the addition of serum or any macromolecular preparation. At confluence, each bottle yielded approximately 2 x 10⁶ cells (L⁻¹ cm⁻²).

Preparation and Characterization of Membrane Vesicles—Membrane vesicles were prepared by a procedure modified from that used by McKeel and Jarrett (21) which we have previously described in detail (18). The purity of the plasma membranes were evaluated by marker enzyme analysis and by enrichment of transport (18).

Determination of Intravesicular Volume—Plasma membrane suspensions containing about 3 mg of protein were centrifuged at 20,000 x g for 20 min. The pellet was resuspended in 0.25 M sucrose/10 mM Tris-HCl, pH 8.0. The volume and protein content of the suspension were determined as follows. The difference between the volume of the suspension and that of medium used for making the suspension was taken as the total vesicular volume. An aliquot of the suspension was incubated at 37°C with [¹⁴C]inulin for 30 min and then centrifuged at 20,000 x g for 20 min. The supernatant was collected, and the pellet was resuspended in the same sucrose/Tris-HCl solution. Because the membrane is impermeable to inulin, the radioactivity in the final membrane suspension is a measure of the extravesicular space. Thus, from the radioactivity in the membrane suspension and that in the supernatant, the extravesicular volume can be derived. By subtracting the extravesicular volume from the total vesicular volume, the intravesicular volume is obtained.

Transport Assay—The reaction mixture, in a total volume of 0.1 ml, consisted of 50 mM potassium phosphate (pH 7.5), 0.1 M NaCl, and 40 to 200 μg of plasma membrane protein. After a preincubation time of 5 min at 37°C, the reaction was started by the addition of L-¹⁴C nucleoside to a final concentration of 8 to 10 μM. The reaction was terminated by addition of 1 ml of prewarmed 0.8 M NaCl solution, followed by filtration under vacuum on nitrocellulose filters (0.3-μm pore size, Millipore). The filters were immediately washed twice with 1 ml portions of warm 0.8 M NaCl solution and dried, and the radioactivity was monitored in a gas flow counter (Nuclear Chicago) at room temperature at 18% counting efficiency. A zero-time value was obtained by diluting the incubation mixture with 1 ml of 0.8 M NaCl before addition of the labeled nucleoside. This zero-time value, which was usually about 1/20 to 1/25 of that of a 10-min incubation mixture, was subtracted from all the corresponding experimental points.

Other Analytical Methods—Assay of (Na⁺,K⁺)-ATPase activity (EC 3.6.1.3), 5'-AMPase activity (EC 3.1.3.5); and NADH diaphorase activity (EC 1.6.99.5) were conducted as described by Avruch and Wallach (22). Succinate-cytochrome c reductase (EC 1.3.99.1) was assayed as described by King (23). Protein was determined according to Lowry et al. (24), using bovine serum albumin as standard.

**RESULTS**

**Differentiation between Plasma Membrane I and Plasma Membrane II**—The final step of the plasma membrane purification procedure is a linear Ficoll gradient centrifugation step which separates the mitochondrial fraction from the plasma membrane (18). The mitochondria sediment to form a pellet, while the plasma membrane equilibrates within the gradient, forming two distinct bands (18). Table I compares physical and enzymatic properties of these two plasma membrane fractions, which we have designated PM I and PM II. The two plasma membrane enzyme markers appear differentially enriched in these two fractions, which contain less than 2% contamination by mitochondrial or endoplasmic reticulum membranes (cf. Ref. 18). Thus, mosaicism for the plasma membrane is suggested. Both membrane fractions exhibit transport activity for the uptake of nucleoside radioactivity, though PM II usually exhibits a specific activity for uptake that is about 40% greater than that of PM I. Out of 11 preparations, however, one was found to have a PM I fraction about 60% more active than the corresponding PM II. Two preparations had PM I fractions which were more active in uptake of radioactivity from inosine, and PM II fractions more active in uptake of radioactivity from adenosine and uridine. For consistency, the experiments presented in this and the accompanying paper were all conducted with PM II.

The plasma membrane fractions prepared by the present procedure are relatively stable when stored below -60°C. Gradual loss of activity has been observed when the membrane preparation was stored at -20°C. After storing at this temperature for 2 months, only about 50% of the original activity was retained.

The ability of the plasma membrane fraction (PM II), the microsomal fraction, and the mitochondrial fraction, (18) to accumulate nucleoside radioactivity from adenosine and inosine is shown in Fig. 1. In both cases, the low transport activities seen for the microsomal and mitochondrial fractions could reflect contamination by plasma membrane vesicles. Approximately 10% of the plasma membrane is trapped in each of these fractions, as calculated from recovery of (Na⁺, K⁺)-ATPase and 5'-AMPase activities (18).

**Kinetics of Plasma Membrane Transport**—When the transport assay is conducted as described under "Experimental Procedure," uptake is linear for at least 2 min when inosine is the substrate, and for at least 4 min when adenosine, guanosine, or uridine is the substrate. The course of uptake of nucleoside radioactivity for these four substrates is shown in Fig. 2. Fig. 3 shows that the rate of uptake is linear over a 25-fold range of membrane concentration. Fig. 4 shows that the transport processes are saturable and obey conventional Mi-
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Michaelis-Menten kinetics. The $K_m$ values extrapolated from the double reciprocal plots are $7.1 \pm 3.5 \times 10^{-6}$ M for adenosine; $5.3 \pm 6 \times 10^{-5}$ M for inosine; and $6.9 \pm 3.5 \times 10^{-5}$ M for uridine. The $K_m$ values have been found to be relatively constant from preparation to preparation. The values for $V_{\text{max}}$, however, vary from batch to batch of membranes, depending on the length of storage of the preparation and the cell density of the culture from which the membrane vesicles were isolated. In general, a preparation obtained from actively growing cells (low cell density) exhibits a higher $V_{\text{max}}$ while freshly prepared vesicles transport better than after storage.

**Effect of pH and Heat on Uptake**—The optimum pH range of the transport systems for which the three nucleosides are substrates is identical and broad. All three systems are fully active from pH 7.0 to 8.8 (potassium phosphate buffer). These transport systems are sensitive to acid environments; they lose 50% of their activity between pH 7.0 and pH 6.5, and only 8% of the activity remains at pH 5.5. These results are shown in Fig. 5. As shown in Table II, heating the vesicles to 52°C after 2 min seems not to significantly affect the transport function for accumulation of radioactivity from purine nucleosides, while a substantial reduction in the ability to accumulate radioactivity from uridine is observed. Heating for 2 min to 63°C reduces all three transport activities; the greatest inhibition is in the membranes' ability to utilize uridine.

**Effect of Inhibitors on Uptake**—Fig. 6 shows that N-ethylmaleimide inhibits all three transport systems. The uptake systems for the two purine nucleosides seem to be affected by

Fig. 2. Time courses of the uptake of radioactivity from adenosine, inosine, guanosine, and uridine by membrane vesicles. The final concentrations of the radioactive nucleosides used were 8.5 µM, 10 µM, 10 µM, and 8 µM for adenosine, guanosine, inosine, and uridine, respectively. Other conditions were as described under "Experimental Procedure." All points represent averages of values from duplicates. O, adenosine; ●, inosine; △, uridine; and □, guanosine.

Fig. 3. Effect of membrane concentration on uptake of radioactivity from adenosine and inosine. The assays were performed according to the procedure described under "Experimental Procedure" except that the concentrations of the radioactive nucleoside used varied as indicated. The incubation time was 5 min for adenosine and 1.5 min for inosine. All points represent the averages of two separate sets of assays. ●, uptake of adenosine radioactivity; O, uptake of inosine radioactivity.

Fig. 4. Double reciprocal plots of the initial rates of the transport of nucleoside radioactivity. The values in the three plots were derived from the experiments shown in the appropriate insets. These experiments were done according to the procedure described under "Experimental Procedure" except that the concentrations of the radioactive nucleosides used varied as indicated. The incubation times were 5, 1.5, and 10 min for adenosine, inosine, and uridine, respectively. All points represent average values obtained from three separate sets of determinations.
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**Fig. 5 (left).** Effect of pH on uptake of nucleoside radioactivity. The measurements were carried out according to the conditions detailed in the legends to Figs. 2 to 4 except that the pH values of the incubation mixtures varied as indicated. For each transport system, the value obtained at pH 7.4 was chosen as $V_0$. All values represent averages from two sets of determinations. $O$, uptake of adenosine radioactivity; $\Delta$, uptake of uridine radioactivity; $\bullet$, uptake of inosine radioactivity.

**Fig. 6 (center).** Effect of N-ethylmaleimide (NEM) on transport of nucleoside radioactivity. The concentration of N-ethylmaleimide in the incubation mixture was as indicated. Other conditions were the same as those described in the legends to Figs. 2 to 4. All values represent averages from two separate sets of determinations. Upper panel shows effect of N-ethylmaleimide on uridine radioactivity uptake. Lower panel shows effect of N-ethylmaleimide on adenosine ($O$); and on uptake of inosine ($O$).

**Fig. 7 (right).** Effect of adenosine on the transport of radioactivity from inosine and uridine. The concentration range of adenosine used was as indicated. Other conditions were the same as specified in Figs. 2 to 4. All points represent values averaged from duplicate assays. $O$, uridine as substrate; $A$, inosine as substrate.

**Table II**

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Transport of nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
</tr>
<tr>
<td>$37^\circ$, 2 min</td>
<td>100</td>
</tr>
<tr>
<td>$52^\circ$, 2 min</td>
<td>86</td>
</tr>
<tr>
<td>$63^\circ$, 2 min</td>
<td>44</td>
</tr>
</tbody>
</table>

N-ethylmaleimide similarly; uridine handling apparently is inhibited to a greater extent.

As shown in Fig. 7, adenosine inhibits the uptake both when inosine and uridine are substrate, however, the effect appears different, especially in the concentration range 0.01 to 0.05 mM adenosine. The converse experiment, the effect of uridine and inosine on the accumulation of radioactivity from adenosine, is shown in Fig. 8. At 0.5 mM, inosine reduces uptake of radioactivity from adenosine virtually to zero. On the other hand, uridine is without effect up to a concentration of 2.0 mM; at this concentration, uridine is in 200-fold excess of the adenosine used. (A 25% reduction of uptake of radioactivity from adenosine has been observed when uridine is present at 10 mM.) Therefore, while adenosine significantly inhibits the uptake of radioactivity from uridine, reciprocal inhibition is not observed. This is in contrast to the adenosine and inosine systems, which are mutually inhibitory. This lack of reciprocality between uridine and adenosine cannot be explained by different affinities of the two nucleosides for a single carrier because the $K_m$ values for the individual systems are nearly identical. A possible explanation for the above observations is that while adenosine at higher concentrations (>$0.05$ mM) may also utilize the membrane component involved in the uptake of radioactivity from uridine, this component is different from the protein(s) involved in the uptake of radioactivity from adenosine and inosine. The heat treatment results shown in Table II are also consistent with this interpretation.

With the exception of inhibition by N-ethylmaleimide (Fig. 6), results shown in Table II and in Figs. 7 and 8 do indicate a possible difference between the systems responsible for the uptake of radioactivity from adenosine and inosine. The same pattern is also seen when 6-methylaminopurine riboside and caffeine (1,3,7-trimethyl purine) are used as effectors in the
two uptake systems (Figs. 9 and 10, respectively). Thus, the
difference, albeit a small one, is expressed in a variety of
circumstances. It will be shown in the accompanying paper
that deamination appears to be a required step in the mem-
bane processing of adenosine (17). This could then explain the
 differences (only one requires deamination) and similarities
(both are processed as inosine) in the two systems.

**DISCUSSION**

This paper and the accompanying one (17) describe the
ability of plasma membrane vesicles isolated from mouse
fibroblasts grown on defined medium to accumulate in-
travesicularly radioactivity from nucleosides. In this paper,
evidence is presented that the plasma membrane vesicles can
be subfractionated into two populations, each possessing
different volumes, equilibrium densities, and enzymatic activi-
ties. When, for consistency, one of these populations was
studied for the ability to take up radioactivity from nucleo-
sides, it was found that uptake was linear with time and
membrane concentration, showed Michaelis-Menten kinetics,
was acid-sensitive, and could be inhibited by a variety of
effectors. The chromatographic analysis of the vesicle contents
after such uptake showed the intravesicular product to be
largely ribose-1-P (17). In the accompanying paper (17), the
relationship of enzymes co-purifying with the membrane,
which might account for the metabolism by the vesicles concomitant to uptake, is examined. Because of the observation,
detailed in the accompanying paper, that nucleosides
themselves do not significantly appear in the vesicles, we have
referred to the transport process in both papers as “uptake of
radioactivity from nucleosides” rather than “nucleoside up-
take,” which, though a less cumbersome phrase, might be
misleading.

One point of interest in this paper is the separation of the
two plasma membrane markers, (Na⁺,K⁺)-ATPase and 5'-
AMPase, in the membrane preparations obtained by the
present procedure. Similar findings in chick embryo muscle
cells have been reported by Schimmel et al. (25). These authors
have also shown that when the cells were first labeled with
¹²⁵I-α-bungarotoxin, which binds to the surface of cultured
muscle cells, the distributions of bound α-bungarotoxin and
(Na⁺,K⁺)-ATPase activity were nearly identical.

It has recently been established that 5'-AMPase is located on
the extracellular surface of the plasma membrane in granulo-
cytes (26). We have been able to obtain two populations of
vesicular membrane fragments from the cell surface, one
enriched for the (Na⁺,K⁺)-ATPase, and the other enriched for
5'-AMPase. The fact that there is some overlap in the activities
observed may reflect the continuum of heterogeneity exhibited
by such plasma membrane vesicles. Wallach and Kamat (21)
and Wallach and Lin (28) have discussed this heterogeneity
both in terms of membrane structure as a mosaic of specialized
functional domains and in terms of the difficulties in purifying
plasma membranes of a heterogenous nature.

In earlier studies it was found that when mouse tumor cell
plasma membrane vesicles (obtained by nitrogen cavitation)
were equilibrated on linear Ficoll gradients, numerous bands of
material were observed in the gradient (19). When such
extensive subfractionation occurs, as it is more likely to occur in
the cavitation process, which results in somewhat smaller
vesicles, than in the present mechanical homogenization
method, certain technical problems are encountered. Although
the ability to observe numerous discrete bands is of great
interest, the quantity of material in each fraction is so small as
to often preclude extensive characterization, especially for a
function such as transport, for which considerable material is
required. In the present study, in which only two fractions were
obtained, we were able to investigate the transport properties
of each fraction. Both fractions PM I and PM II were functional
in transport for the nucleoside substrates used. Quantitative
differences did appear in the $V_{max}$ values between different
batches of vesicles and also between PM I and PM II. We felt
that rather than a superficial inquiry into the quantitative
differences, it was important to try to elucidate a transport
mechanism in one fraction and then to compare mechanisms
between the fractions. Thus, further evaluation of nucleoside
handling and the uptake by PM II is the subject of the
accompanying paper (17), while a comparison of the nucleoside
processing in PM I and PM II must await future investigation.

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   Acad. Sci. U. S. A.* 70, 3631-3635

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Unpublished results.*
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