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Two-Compartment Behavior During Transport of Folate Compounds in L1210 Cell Plasma Membrane Vesicles

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Summary. The transport of [^{3}H] 1,L 5-formyltetrahydrofolate, [^{3}H] folic acid, and [^{3}H]methotrexate by L1210 cell plasma membrane vesicles exhibited multicompartmental behavior. Two separate vesicular compartments (parallel relationship) of approximately equal volume were revealed during measurements of influx and efflux. Flux in one compartment was rapid, saturable, highly temperature-sensitive, and inhibited by pCMBS. Flux in the other compartment exhibited all of the characteristics of passive diffusion. These results imply that our plasma membrane vesicle preparations consist of a mixture of two functional species. Transport of folate into one of these species occurs by passive diffusion alone, whereas transport into the other kind of vesicle occurs by both passive diffusion and carrier-facilitated transport.

Key words folate transport · plasma membrane vesicles · L1210 cells

Introduction

The isolation of plasma membrane vesicles from various prokaryotic and eukaryotic cells has provided [7, 8, 21] a very useful method for studies of membrane transport without the complication of intracellular metabolism. Consequently, studies have been carried out with this system which address a variety of biological questions or probe aspects of plasma membrane structure and function. In our own studies [23], we have used these vesicle preparations for examining transport of folate analogs by tumor cells. Membrane vesicle preparations from L1210 leukemia cells were found to be competent for carrier-mediated transport by a system using reduced folate coenzymes and folate analogs as substrates. However, the data derived were also suggestive of a complexity not ordinarily seen in intact L1210 cells. During both accumulation and exit of [^{3}H]methotrexate the time-course exhibited bi-exponential kinetics. Viewed in the light of other data also presented, these results appeared to be characteristic of multi-comparmental behavior. We now present additional data derived from biochemical studies and a more detailed kinetic analysis of transport of a variety of folate compounds by these membrane preparations which consistently revealed the existence of two separate vesicular compartments in a proportion of approximately 1:1. Although the kinetic analysis does not, by itself, distinguish between a series or parallel relationship for each vesicular compartment with respect to the external compartment, electron microscopy done on these preparations supports the notion of a parallel relationship. Although, the physical basis of this heterogeneity is not completely explained by the data, each compartment appears to represent a separate subpopulation of vesicles of which only one is competent for carrier-mediated transport. Both compartments, however, are accessible to the various folate compounds by simple diffusion. The data also appears to exclude the possibilities that the incompetent subpopulation is contaminated by other membranes from organelles or inside-out vesicles. Moreover, the same degree of heterogeneity is observed following different methods of vesicle preparation. Since there is evidence from other studies by us and in the literature [3–5, 9] for similar complexity during measurements of transport of a variety of substrates by vesicles prepared in a similar fashion from different cell types, the findings presented here may have broad biochemical implications.

Materials and Methods

Materials

[^{3}H] d1,L 5-formyltetrahydrofolate ([^{3}H] 5-formylfolate-H_4) with a specific activity of 3 Ci/mmol and [^{3}H]methotrexate with a specific activity of 20 Ci/mmol were purchased from Moravek Biochemicals, City of Industry, California. [^{3}H] Folic acid with a specific activity of 5 Ci/mmol and 3-O-[^{14}C]-methyl-D-glucose with specific activity of 58.9 mCi/mmol were purchased from

0022-2631/82/0068-0019 $02.00

Amersham/Searle, Chicago, Illinois. [^{3}H] Inulin with a specific activity of 0.48 mCi/mg was obtained from ICN Pharmaceutical Co., Irvine, California. [^{14}C] inulin (sp. act. of 2.04 mCi/g) and [^{3}H]$_2$O (sp act of 1 mCi/g) were purchased from New England Nuclear, Boston, Mass. Ultrapure sucrose (Schwarz/Mann) was used for preparing the density gradients. Methotrexate and dl,L 5-formyltetrahydrofolate were provided by Dr. Harry B. Wood, Jr., Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. dl,L 5-methyltetrahydrofolate was obtained from Sigma Biochemicals as the barium salt and converted to the sodium salt by a method already described elsewhere [15]. [^{3}H] dl,L 5-formylfolate-H_4 and [^{3}H] folic acid was purified prior to use by DEAE-cellulose chromatography [13]. Radiochemical purity of the final preparation was >97%. Aliquots of purified material were stored at −70°. All other chemicals were reagent grade.

Methods

Source of Cells: L1210 leukemia cells were obtained by transplantation [14] in $BD2F_1$ mice of a cell line ($L1210/V\text{-}C_1$) established in culture. Ascites suspension was harvested in cold (0°) buffered isotonic saline (0.14 M NaCl + 0.01 M sodium phosphate, pH 7.4) from the peritoneal cavity 5–6 days after transplantation. Contaminating red blood cells were removed by repeated washing of the tumor cell suspension with cold (0°) 0.17 M NH_4Cl [12] and then with 0.14 M NaCl.

Plasma membrane isolation: Two procedures were employed during the course of these studies: (A) The method of Tsai et al. [18] was used with some modifications as previously described [23] for the isolation at 0° of L1210 cell plasma membranes following mechanical disruption of washed cells while suspended in 10 mM sodium phosphate (pH 7.0) containing 0.9% NaCl, 1 mM $CaCl_2$, 1 mM MgI_2 and 0.5 M hexylene glycol. The final washed membrane pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 150 mM sucrose and stored at −70° until used. (B) A modification of the method developed by Hochstadt et al. [7] using nitrogen cavitation [22] for cell disruption was also employed while maintaining a temperature of 4°. Cells in the same homogenizing buffer (*see* above) were equilibrated 20 min at 600 psi of nitrogen while stirring in a pressure homogenization apparatus (Parr Instruments, Moline, Illinois). Cell breakage was monitored under phase contrast microscopy. Nuclei remained intact and were removed along with other organelles and remaining intact cells by centrifugation at 3000 × g for 10 min. Discontinuous sucrose gradient centrifugation for plasma membrane isolation and storage of the final preparation were the same as described in Method A. A determination of purity of membrane preparations by marker enzyme analysis and electron microscopy has been described [23].

Transport assay: Membrane suspensions were equilibrated for 5 min at the required temperature, and the experiment was initiated by adding [^{3}H] 5-formylfolate-H_4 [^{3}H]methotrexate or [^{3}H] folic acid to a final volume of 0.1 ml in transport buffer containing 50 mM potassium phosphate (pH 7.4), 150 mM sucrose and 100 mM NaCl. The membrane protein concentration in different experiments varied from 0.1 to 0.4 mg per tube. Incubation was terminated by 20-fold dilution with 0.8 M NaCl and then immediately filtering through 25 mm microporous filters (0.2 micron, Amicon). After washing three times with 0.8 M NaCl, the filter was dried in air and the radioactivity remaining measured in Hydrofluor (National Diagnostics) by scintillation spectrophotometry with a counting efficiency of 30% for ^{3}H in the presence of the filter. Loss of intravesicular tritium by passage of intact vesicles through the filter or by disruption amounts to approximately 10% of the total tritium. This was determined from the recovery of radioactivity in vesicles washed by centrifugation *versus* filtration and from a measurement of the protein present in the eluate after filtration. Concentrations for L-5-formyltetrahydrofolate and L-5-methyltetrahydrofolate are expressed as the natural (*l*) diastereoisomer. Previous studies [18] from our laboratory have shown that the high affinity carrier system in intact L1210 cells exhibited, during influx, stereospecificity at carbon-6 of this folate coenzyme. From data on competition experiments with both natural and unnatural (D) isomers of 5-formyltetrahydroflate reported at that time, we would conclude that uptake of the unnatural isomer by this system would amount to less than 4% of the total carrier mediated uptake. However, both natural and unnatural isomers would be expected to accumulate by passive diffusion.

Efflux of [^{3}H] 5-formylfolate-H_4, [^{3}H]methotrexate and [^{3}H] folic acid was studied by preloading the vesicles with each compound for 20 min at 37°. The vesicle preparation was diluted with cold (0°) transport buffer and collected by centrifugation at 23,000 × g for 30 min. After washing once with the same buffer, the final pellet was again resuspended in transport buffer (extravesicular/intravesicular volume = 100–200) and aliquots were removed at varying times after incubation was initiated and processed as described above.

Chromatographic analysis: Intravesicular [^{3}H] dl,L 5-formyltetrahydrofolate, [^{3}H]methotrexate and [^{3}H] folic acid which accumulated during a 30 min incubation (37°) of 2 μM of each with the vesicle preparations chromatographically [13] were analyzed on DEAE-Sephadex (Pharmacia) along with aliquots of the original samples. After incubation the vesicle suspension was washed three times and heat treated (100°) in 2% sodium ascorbate to release substrate. Aliquots were brought to an ionic strength of 0.3 M NaCl with 1 M NaCl and applied to a DEAE-Sephadex A-25 column (0.9 × 30 cm). Following an initial wash of 20 ml of 0.3 M NaCl in phosphate buffer (0.05 M potassium phosphate, pH 7.0), the samples were eluted with a linear gradient of 0.3 to 0.5 M NaCl in phosphate buffer, collecting 2 ml fractions at a flow rate of 8–10 ml/hr. Radioactivity of a 1-ml aliquot of each fraction in 10 ml of Scintisol (Isolab) was determined by scintillation spectrophotometry.

Assay for bound substrate: Plasma membrane vesicles were incubated for 30 min at 37° with 2 μM of each of the following substrates, [^{3}H]methotrexate, [^{3}H] 1, L 5-CHO-folate H_4 and [^{3}H] folic acid and then washed three times with transport buffer by centrifugation at 35,000 × g for 30 min at 4°. The final washed plasma membranes were solubilized [16] by adding 1% (vol/vol) of triton X-100 and centrifuging as before. The supernatant fraction with unlabeled substrate as marker was applied to a Sephadex G-25 column (0.9 × 25 cm) at room temperature and eluted with 0.3 M NaCl plus 50 mM potassium phosphate (pH 7.0). Blue dextran was used to identify the void volume. 1-ml fractions were collected, and radioactivity was determined by scintillation spectrophotometry.

Other analytical methods: Measurements of intravesicular and extravesicular volume by double-labeling with [^{3}H] inulin and [^{14}C]-3-O-methyl-D-glucose were made by the method of Hissin and Hilf [6] or by a method [10] using [^{3}H]$_2$O and [^{14}C] inulin. Values derived from an average of four determinations on different preparations was 2.47 ± 0.06 μl per mg membrane protein for preparations made by method A and 4.15 ± 0.3 μl per mg membrane protein made by method B. The difference in these values reflects at least partially the purity of each preparation. It may also reflect the relative amount of membrane in each case which exists in a vesicular form. Electron microscopy of the

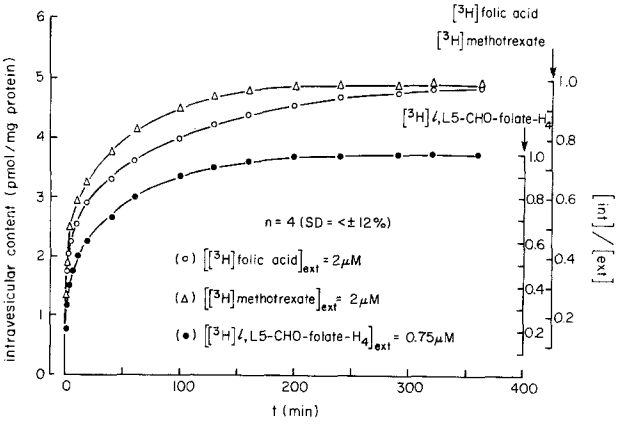

Fig. 1. Time-course for intravesicular accumulation at 37° of folate compounds. Membrane preparations were equilibrated for 5 min, and the experiment was initiated by the addition of each folate to the transport buffer. Final volume was 0.1 ml. Incubation was stopped by dilution and filtration, for which details are provided in the text. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H_4; [^{3}H] 1,L 5-formyltetrahydrofolate

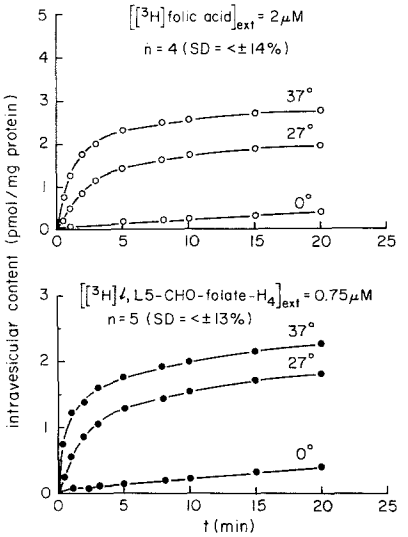

Fig. 2. Time-course for initial intravesicular accumulation of folate compounds. Membrane preparations were equilibrated for 5 min at each temperature, and the experiment was initiated by the addition of each folate to the transport. Additional details are provided in the legend of Fig. 1 and in the text. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H_4; [^{3}H] 1,L 5-formyltetrahydrofolate

membrane preparations has been carried out by procedure described [2] elsewhere by other workers.

Results

Intravesicular Accumulation of Folate Compounds

Data on the time-course at 37° for accumulation to steady state is shown for [^{3}H] 1,L 5-formyltetrahydrofolate, [^{3}H] folic acid and [^{3}H]methotrexate in Fig. 1. The data shown here and in the other figures has been corrected for tritium (0.1–0.2 pmol substrate/mg membrane protein) associating with washed vesicles after 10 sec incubation at 0° which is presumed to represent surface adsorption [14, 23]. From an examination of the data it can be seen that intravesicular accumulation exhibited a complex time-course, i.e., uptake was characterized by an initial rapid phase followed within 1 to 2 min by a slow phase which continued until steady state was reached. The two phases are seen more clearly in Figs. 2 and 3 where only the initial time-course for accumulation of each substrate was compared. Data are also shown for accumulation of each substrate at 27 and 0°.

One possible explanation for the complexity of the time-courses shown in Figs. 2 and 3 is that the slow uptake component represents metabolism and (or) binding of intravesicular substrate. To test this explanation, aliquots of the vesicle preparations were incubated at 37° for 30 min with 2 μM of each radiolabeled substrate. The suspensions were then cooled (0°), washed, and heat-treated (100°) to extract substrate. After clarification of the sample by

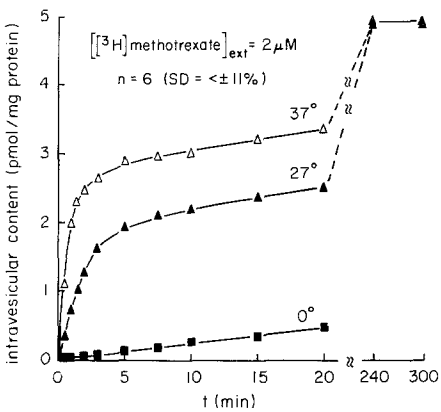

Fig. 3. Time-course for initial and steady-state level of intravesicular accumulation of [^{3}H]methotrexate. Membrane preparations were equilibrated for 5 min at each temperature, and the experiment was initiated by the addition of substrate. Additional details are given in the legend to Fig. 1 and in the text

centrifugation, the supernatant was chromatographed by means of an anion exchange column. From a comparison of the elution profile of the material with that of the original sample, it was found in the case of each substrate that >97% of the radioactivity had elution properties equivalent to the original sample. In a related experiment, aliquots of vesicle suspension incubated at 37° for 30 min with 2 μM of each substrate were cooled (0°), washed, and solubilized with Triton X-100. After centrifugation, the supernatant was chromatographed on Sephadex G-25. From data on the recovery of radioactivity in various eluted fractions, the relative

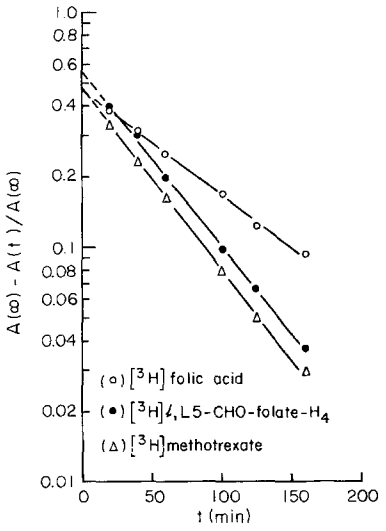

Fig. 4. Decay-time analysis of data on the time-course for intravesicular accumulation at 37° of folate compounds. Experimental details are given in the legend of Fig. 1 and in the text

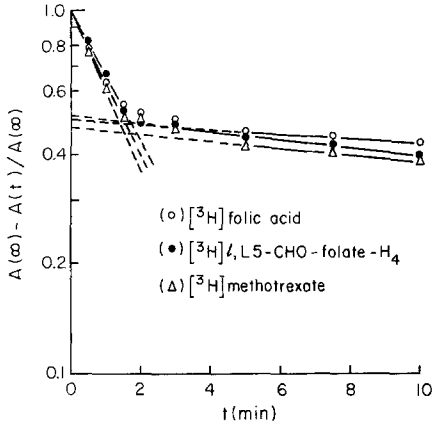

Fig. 5. Decay-time analysis of data on the initial time-course for intravesicular accumulation at 37° of folate compounds. Experimental details are given in the legend of Fig. 1 and in the text

amount of radioactivity found associated with the void volume, where high molecular weight proteins are excluded, was 1.6, 2.3 and 14.6% for [^{3}H] 1,L 5-formyltetrahydrofolate, [^{3}H]methotrexate, and [^{3}H] folic acid, respectively. The majority of the radioactivity in each case was associated with the elution volume equivalent to the original substrate. An explanation assuming slow denaturation or decay of components (e.g., carrier molecules) also appeared to be excluded, since a delay in addition of substrate for as long as 20 min, had no effect on the time-course for accumulation.

In view of the above results it seemed likely that the time-course data could be explained as multicompartmentation; that is, a portion of the intravesicular volume was more accessible to substrate than the remaining volume.

It is generally understood from the theory of compartmental analysis [11] that the time-course for accumulation or loss of label in some part of an n compartment system, in which total label is conserved, is given by the sum of a constant and $n-1$ exponential terms

$$A(t)=A(\infty)+A_1 e^{-\lambda_1 t}+A_2 e^{-\lambda_2 t}+\ldots+A_{n-1} e^{-\lambda_{n-1} t} \quad (1)$$

where $A(\infty)$ is the constant term and the terms A_i are constants representing the amplitudes of the exponential components. This theorem cannot be applied without reservation in the case of carrier mediated transport, as in the present case [14, 23], because compartment analysis assumes that there are no nonlinear (i.e., saturable) terms in the fundamental equations for transport. Nevertheless, if the concentration of substrate is sufficiently small so that mediated transport is not saturated, the theorem will hold to a good approximation. The theorem will also hold even when mediated transport is saturated, provided the ratio of the extravesicular to intravesicular concentration is not too far from steady-state so that the transport equations can be linearized.

Figures 4 and 5 show semilog plots of $A(\infty)-A(t)$ for accumulation of low levels of each of the three substrates. It can be seen that the data are represented by the sum of a constant term plus two exponentials. This delineates two intravesicular compartments with respect to a third compartment representing the extravesicular space. This latter compartment is of such large relative volume ($>100:1$) that the associated concentration of the folate compound can be regarded as constant.

The data presented in Fig. 1 show that accumulation of the various folate compounds is equilibrative with respect to the extravesicular compartment. This conclusion is derived from values for distribution ratio ([int]/[ext]) at steady-state which approximates unity for each substrate. Also, from data derived in a related experiment, steady-state levels of accumulation for different concentrations of each substrate, as in the case of [^{3}H] 1,L 5-formyltetrahydrofolate (Fig. 6) show a linear relationship with concentration over a 20-fold range.

In light of the evidence that transport in these vesicle preparations is equilibrating, two alternative compartment models can be proposed to explain the two components of transport of these folate compounds. The simplest possibility is that there are two separate populations of membrane vesicles (parallel relationship) with differing characteristics for transport. The second model assumes that a fraction or all of the vesicles have a second vesicle within the

intravesicular space (series relationship). Although the second model is least likely to apply, it is not possible to make a rigorous kinetic discrimination between the two models. The physical evidence provided by electron microscopy, however, appears to support the idea that it is a parallel rather than series relationship. As reported in our prior study [23], the vesicles appear as empty sacs varying from 0.1 to 0.4 μm in radius.

Assuming that two separate populations of membrane vesicles leads to compartment equations of the form

$$\frac{d}{dt}m_1 = p_1 s_1 (c_{ex} - m_1/v_1) \tag{2a}$$

$$\frac{d}{dt}m_2 = p_2 s_2 (c_{ex} - m_2/v_2). \tag{2b}$$

In these equations m_1 and m_2 are the masses of folate in pmol/mg protein in the two vesicle populations and c_{ex} is the constant concentration of folate in the external compartment. The terms, v_1, v_2, s_1 and s_2, represent the volumes and surface areas of the two compartments (cm^3 and cm^2 per mg membrane protein, respectively). The terms p_1 and p_2 represent the permeabilities per unit surface area (cm/sec) of the two compartments; p_1 and p_2 reflect the molecular structure of the membrane of the two populations and are thus of primary interest.

It is important to realize that Eqs. (2a) and (2b) result from linearization of the full nonlinear transport equations. As a result of this approximation both carrier mediated and diffusive transport are lumped together into the "effective" permeability coefficients p_1 and p_2.

To illustrate, if folate enters compartment 1 by both simple diffusion and facilitated diffusion then the full nonlinear transport equation for compartment 1 is of the form

$$\frac{d}{dt}m_1 = s_1 p_{d1}(c_{ex} - m_1/v_1) + \frac{s_1 p_{c1}[c_{ex} - m_1/v_1]}{(1 + j_1 c_{ex} + j_2 m_1/v_1 + j_2 c_{ex} m_1/v_1)}. \tag{3}$$

In this equation p_{d1} represents the diffusion permeability of compartment 1, p_{c1} represents the maximum permeability due to carrier mediated transport in this compartment ($V_{max} = p_{c1} s_1/j_1$); $j_1 j_2$ and j_3 are positive constants related to the binding of carrier on the inside and outside of the membrane.

Equation (3) is highly nonlinear and difficult to directly analyze. The analysis can be simplified by expanding the right-hand side of this equation in

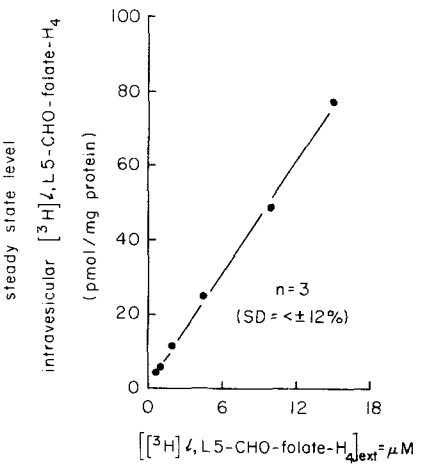

Fig. 6. Steady-state level of intravesicular accumulation at 37° following incubation with different concentrations of [^{3}H] 1,L 5-formyltetrahydrofolate. *See* legend of Fig. 1 and text for additional details. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H_4; [^{3}H] 1,L 5-formyltetrahydrofolate

Taylor's series about the steady-state value $m_1 = c_{ex} v_1$. When this is done we obtain

$$\frac{d}{dt}m_1 = s_1[p_{d1} + p_{c1}/(1 + (j_1 + j_2)c_{ex} + j_3 c_{ex}^2)] \cdot (c_{ex} - m_1/v_1) + \text{higher order terms}. \tag{4}$$

Neglecting the higher order terms we see that Eq. (4) is of the same form as Eq. (2a), and the lumped permeability coefficient is given by

$$p_2 = p_{d1} + p_{c1}/(1 + (j_1 + j_2)c_{ex} + j_3 c_{ex}^2). \tag{5}$$

Equation (5) shows that if transport into a compartment involves a significant amount of facilitated diffusion, then the lumped permeability will decrease as the external concentration is raised. Ultimately, at very high external concentration, diffusion will become the dominant mode of transport.

The solution to Eqs. (2a) and (2b) subject to the arbitrary initial conditions, $m_1(0) = m_{10}$ and $m_2(0) = m_{20}$ is

$$m_1(t) = v_1 c_{ex} + [m_{10} - v_1 c_{ex}] e^{-\lambda_1 t} \tag{6a}$$

$$m_2(t) = v_2 c_{ex} + [m_{20} - v_2 c_{ex}] e^{-\lambda_2 t} \tag{6b}$$

where

$$\lambda_1 = p_1 s_1/v_1 \quad \text{and} \quad \lambda_2 = p_2 s_2/v_2. \tag{6c}$$

Notice that because of the parallel relationship between the two compartments λ_1 depends only on the parameters of compartment 1 and λ_2 depends only on the parameters of compartment 2. This implies that in general the larger of λ_1 and λ_2 will designate

Table 1. Properties of fast and slow intravesicular compartments accumulating folate compounds

Substrate	c_{ex} (μM)	Compartment	v[a] (μl/mg)	λ[b] (min^{-1})
[^{3}H] folic acid	2	Fast	1.31	0.4950
	2	Slow	1.16	0.0093
	50	Fast	1.27	0.4720
	50	Slow	1.20	0.0091
[3H] 1,L 5-CHO-folate-H_4	0.75	Fast	1.09	0.9890
	0.75	Slow	1.38	0.0185
	10.0	Fast	1.15	0.4340
	"	Slow	1.32	0.0206
[^{3}H]methotrexate	2	Fast	1.28	0.7720
	2	Slow	1.19	0.0191
	9	Fast	1.32	0.4620
	9	Slow	1.15	0.0182

[a] Total volume is 2.47 ± 0.06 μl per mg membrane protein.
[b] Derived by the method of exponential peeling using data like that shown in Figs. 2 and 6.

Table 2. The effect of temperature on the properties of fast and slow compartments during accumulation of [^{3}H]methotrexate

Temp. (°C)	Compartment	v (μl/mg)	λ (min^{-1})
27	Fast	0.86	0.2540
	Slow	1.61	0.0193
37	Fast	1.27	0.7630
	Slow	1.20	0.0189

a so called "fast compartment" whereas the slower will designate a "slow compartment." Henceforth we will arbitrarily let the fast and slow compartments be compartments 1 and 2, respectively.

In our experimental system the quantities m_1 and m_2 cannot be observed separately since the two observed populations are completely intermixed. The observable quantity is the lumped intravesicular mass of folate

$$m_T = m_1 + m_2 = (v_1 + v_2)\, c_{ex} + (m_{10} - v_1 c_{ex})\, e^{-\lambda_1 t} + (m_{20} - v_2 c_{ex})\, e^{-\lambda_2 t}. \quad (7)$$

Clearly measurements of m_T are sufficient to determine v_1, v_2, λ_1 and λ_2.

In Table 1 a summary is given of the physical quantities v_1, v_2, λ_1 and λ_2 for three folate compounds derived by means of Eqs. (6a), (6b), and (6c) using the data for accumulation at 37° which is shown in Figs. 1 through 5. For all three substrates the time constants for accumulation in the fast compartment is very much greater than in the slow compartment. This is shown by a comparison of the values for $\lambda_1 = p_1 s_1 / v_1$ and $\lambda_2 = p_2 s_2 / v_2$. There was no difference among the three substrates with respect to the size of the compartments. It is interesting that both λ_1 and λ_2 were smaller for [^{3}H] folic acid than for the other two substrates.

Values for v_1 and v_2 varied slightly from day to day, but the ratio between v_1 and v_2 was insensitive to concentration. In the case of [^{3}H] 1,L 5-formyltetrahydrofolate and [^{3}H]methotrexate, λ_1 decreased with increase in external concentration; λ_1 showed no concentration dependence in the case of folic acid. λ_2 showed no concentration dependence for any of the folate compounds studied.

There were large differences in temperature sensitivity of accumulation in each compartment. This is shown in Figs. 2 and 3 and is represented by data on Q_{10} (27°–37°) determined for each component of uptake. For all three folates entry into the fast compartment exhibited a Q_{10} of 3.0–3.7 while entry into the slow compartment exhibited a Q_{10} of unity. The volume ratio between fast and slow compartments was also affected by temperature. In the case of [^{3}H]methotrexate (*see* Table 2) and the other folates (data not shown), the volume of the fast compartment was reduced from about 1/2 to approximately 1/3 of the total vesicular volume. Measurements [10] of intravesicular space at 27° *vs.* 37° showed that total vesicular volume remained the same at either temperature. This was also shown in Fig. 3 from the data on intravesicular accumulation at steady-state at each temperature. This is interpreted to mean that at the lower temperature a partial conversion occurs between fast and slow compartments.

From Eq. (6c) we see that there are two ways to explain the very large difference between $\lambda_1 = p_1 s_1 / v_1$ and $\lambda_2 = p_2 s_2 / v_2$. The simplest explanation is that the difference is a result of a difference in the ratio of surface area to volume of the two vesicle compartments (i.e., $s_1 v_1 \gg s_2 v_2$ but $p_1 \simeq p_2$). If this hypothesis is correct then it is difficult to see why decreasing the temperature from 37 to 27 °C has a threefold effect on λ_1 but no effect on λ_2. A change in temperature should not affect the surface area or volume of the vesicles whereas according to our simple hypothesis, p_1 and p_2 should be affected to the same extent.

As a further test of whether or not the two compartments are due to different sized vesicles, the effect of 200 μM pCMBS on the time-course for accumulation of the various folate compounds was studied. As shown in Fig. 7, the course for accumulation of [^{3}H] 1,L 5-formyltetrahydrofolate and [^{3}H]methotrexate was extremely sensitive (90–95% inhibition) to this concentration of pCMBS. Initial accumulation of [^{3}H] folic acid, however, was some-

what less sensitive (40% inhibition). In sharp contrast to these results, the late phase of accumulation of each substrate was unaffected by pCMBS. Once again this indicates that size is not an important factor in differentiating between the two vesicle populations.

The initial time-course for [^{3}H] 1,L 5-formyltetrahydrofolate accumulation at 37° at different extravesicular (1–20 μM) concentrations was determined (data not shown). At each concentration accumulation was linear with time for an interval of 30 to 60 sec. The same time-course was obtained (data not shown) for initial accumulation at 37° of [^{3}H]methotrexate and [^{3}H] folic acid. From a double-reciprocal plot of influx derived from the initial 30 sec of accumulation *vs.* concentration, a saturable and nonsaturable (or poorly saturable) component of initial influx was delineated. Initial influx of both [^{3}H] 1,L 5-formyltetrahydrofolate and [^{3}H] methotrexate was highly saturable [apparent $K_m = 3\text{-}4 \pm 0.6$ μM ($n=6$)] but exhibited a relatively low apparent V_{max} 3 ± 0.4 pmol/min/mg membrane protein). In contrast to these results, initial influx of [^{3}H] folic acid exhibited low saturability [$K_m = 409 \pm 66$ μM ($n=6$) and an extremely high V_{max} (123 ± 33 pmol/min/mg protein.

Taken by themselves the data on initial influx demonstrate both saturable and nonsaturable uptake, but they tell us nothing about the compartmentalization of this uptake. In order to decide which of the two compartments has the saturable component or whether they both contain this component we must look at the values of λ_1 and λ_2 derived from the entire time course.

As shown by Eq. (3), the presence of a saturable component of transport in a vesicle population should cause the decay rate for that compartment to decrease with external concentration. On this basis the data in Table 1 indicate saturable transport into the fast compartment for both reduced folates. The strong evidence for saturable transport of the reduced folates provided by the initial velocity studies confirms the data in Table 1. Therefore, we conclude that the saturable component of uptake occurs predominantly in the fast compartment (at least for the reduced folates), whereas the slow compartment is nonsaturable. Because of the very low saturability of folic acid transport ($K_m = 409$ μM) we cannot rigorously tell which compartment had the saturable component. However, by analogy with the other folates it is likely that mediation of folic acid flux also occurs only in the fast compartment.

The observation that transport in the slow-nonsaturable compartment is insensitive to temperature or PCMBS leads us to think that it represents passive diffusion. If we accept this conclusion then the data imply that the two-compartment behavior is due to the presence of a considerable fraction of carrier incompetent vesicles. Also, if the slow component represents simple diffusion across the membrane then the fast compartment must transport folate by both carrier mediated and simple diffusion and not carrier mediation alone.

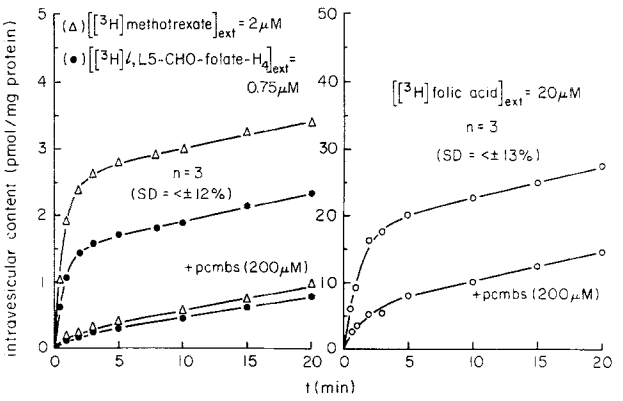

Fig. 7. The effect of pCMBS on the intravesicular accumulation at 37° of folate compounds. The membrane preparations were equilibrated for 5 min at 37° in the presence of pCMBS and substrate was added. *See* text for additional details. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H$_4$; [^{3}H] 1,L 5-formyltetrahydrofolate

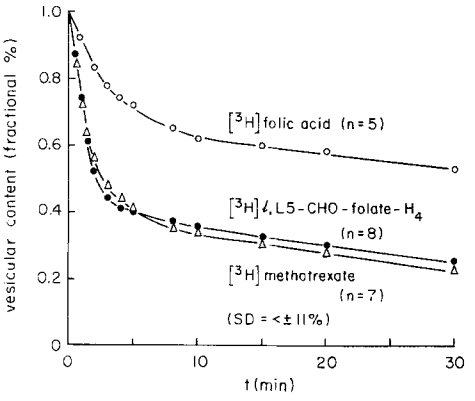

Fig. 8. Time-course for efflux at 37° of intravesicular folate compounds. Vesicle preparations were incubated for 20 min in the presence of 2 μM [^{3}H] 1,L 5-formyltetrahydrofolate or [^{3}H]methotrexate or 50 μM [^{3}H] folic acid, cooled and washed at 0° and reincubated at 37° in transport buffer alone. *See* text for additional details. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H$_4$, [^{3}H] 1,L 5-formyltetrahydrofolate

Efflux of Folate Compounds

The time-course for efflux at 37° of each folate compound is shown in Fig. 8. Vesicles were preloaded by a 20-min incubation in the presence of each compound, washed and then resuspended in their absence. The data given is expressed as fractional per cent of the initial vesicular content at each time point. Efflux of [^{3}H] 1,L 5-formyltetrahydrofolate

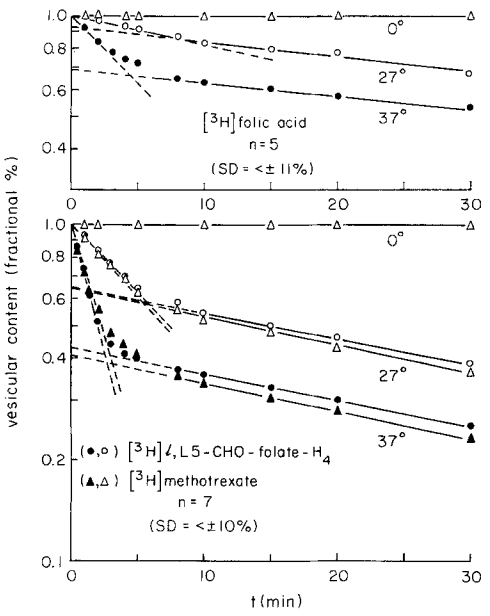

Fig. 9. Decay-time analysis of the time-course for efflux of intravesicular folate at different temperatures. *See* legend of Fig. 8 and text for other details. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H_4; [^{3}H] 1,L 5-formyltetrahydrofolate

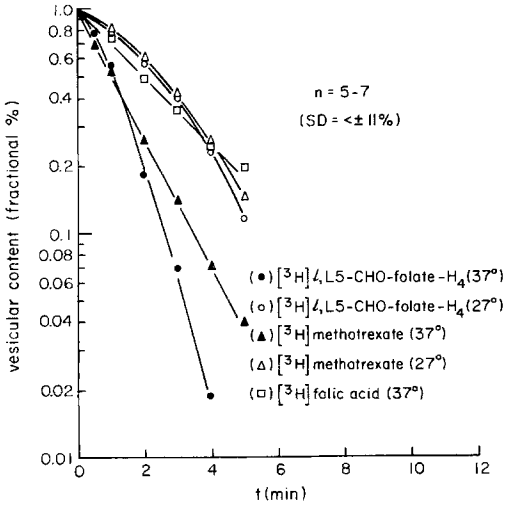

Fig. 10. Decay-time analysis of initial efflux at different temperatures. Data derived by exponential peeling of the data provided in Fig. 9. *See* legend of Fig. 8 and text for additional details. *Abbreviation:* [^{3}H] 1,L 5-CHO-folate-H_4; [^{3}H] 1,L 5-formyltetrahydrofolate

and [^{3}H]methotrexate were more rapid than efflux of [^{3}H] folic acid. This was particularly noticeable during the first few minutes of incubation. A decay-time analysis by semilog plot of relative intravesicular content with time from data on the efflux time-course obtained at 0°, 27° and 37° is shown in Fig. 9. For each substrate two components of efflux with distinctly different values for λ were delineated, a rapid initial component (λ_1) which was highly temperature-sensitive and a slow late component (λ_2) which was relatively temperature-insensitive. During the period of incubation shown there was no detectable loss of any substrate at 0°. A reduction in the size of the fast compartment (v_1) *vs.* slow compartment (v_2) was also obtained at 27° which was similar in magnitude to that seen during influx at this temperature. An analysis of the kinetics of the fast component of efflux of each substrate is shown in Fig. 10. The time-courses shown were derived by "exponential peeling" of the data shown in Fig. 9. At 37° and especially at 27° there was some indication of nonexponential decay for both [^{3}H] 1,L 5-formyltetrahydrofolate and [^{3}H]methotrexate, but not for [^{3}H] folic acid. This supports the conclusion that part of the transport in this fast compartment is saturable.

Table 3. Properties of fast and slow vesicular compartments effluxing folate compounds

Substrate	Loading conditions[a]	Compartment	v^b (μl/mg)	λ^c (min^{-1})
[^{3}H] folic acid	A	Fast	0.74	0.3650
		Slow	1.73	0.0093
[^{3}H]methotrexate	B	Fast	1.38	0.8650
		Slow	1.09	0.0184
[^{3}H] 1,L 5-CHO-folate-H_4	C	Fast	1.66	0.7690
		Slow	0.81	0.0181
	D	Fast	0.86	0.8640
		Slow	1.61	0.0171

[a] A, 20 μM for 20 min; B, 2 μM for 20 min; C, 1 μM for 5 min; and D, 5 μM for 30 min.
[b] Total volume is 2.47 ± 0.06 μl per mg membrane protein.
[c] Derived by the method of exponential peeling using data similar to that shown in Fig. 10.

A summary of the efflux data at 37° is presented in Table 3. Note that the decay rates of the two components of transport (λ_1 and λ_2), as derived from efflux studies, as well as their temperature dependence are the same as that derived from influx studies at low external concentrations. As one would expect, the size of each compartment derived during efflux of each substrate was dependent upon the loading conditions employed. At short periods of incubation with low external concentrations of substrate, the fast compartment was appreciably larger than the slow compartment. At higher external concentrations and longer loading periods the reverse was true. In contrast to these results, the values for λ pertaining to each compartment was indifferent to changes in loading conditions. As in the case of influx (Table 1) values for λ for both fast and slow compartments of [^{3}H] folic acid efflux were smaller than the values derived with the other two compounds.

Table 4. Compartmental analysis of [^{3}H]methotrexate accumulation and exit with membrane vesicles prepared by different methods

Phase[a]	Method[b]	Compartment	v[c] (μl/mg)	λ[b] (min^{-1})
Entry	A	Fast	1.19	0.6930
		Slow	1.28	0.0221
	B	Fast	2.11	0.7120
		Slow	2.02	0.0196
Exit	A	Fast	1.43	0.7640
		Slow	1.04	0.0205
	B	Fast	2.56	0.7420
		Slow	1.57	0.0217

[a] Accumulation at 37° was measured to equilibrium in the presence of 2 μM [^{3}H] methotrexate. Exit at 37° was measured after preloading vesicles at a concentration of 2 μM [^{3}H]methotrexate for 20 min.

[b] A, method of Tsai et al. [18]; B, method of Hochstadt et al. [7].

[c] Derived by the method of exponential peeling using data like that shown in Figs. 2 and 6. Total volume: 2.47 μl/mg protein (Method A); 4.13 μl/mg (Method B).

Comparison of Data on Accumulation and Exit of [^{3}H]methotrexate by Vesicles Prepared by Different Methods

In a parallel series of experiments, measurements of accumulation and exit at 37° of [^{3}H] methotrexate by vesicles derived by two different methods (*see* Materials and Methods) of preparation were carried out. The data was submitted to the same decay-time analysis as that shown in Figs. 4, 5 and 9 and summarized in Table 4. In vesicles prepared by either method, both fast and slow compartments were delineated from data on influx and efflux of this substrate. Moreover, the ratio between the size of each compartment was essentially identical.

Analytical Studies Related to Membrane Orientation

Since the data delineating two distinct vesicle compartments might be explained by a difference in orientation (right-side-out *vs.* inside-out) a determination for surface markers in the vesicle preparation was carried out. The activity of 5′-nucleosidase an ectoenzyme [1] and sialic acid content [21] were assayed in the presence and absence of 0.2% Triton X-100 which solubilizes [*see* ref. 16 and a previous section of the text] the plasma membrane. If a substantial fraction of the vesicles are in a wrong-side-out orientation, then the value obtained in each case in the presence of Triton X-100 should be higher. The values obtained in the absence of Triton (5′-nucleosidase = 630 ± 130 nmol/min/mg protein [n = 7] and sialic acid = 41.7 ± 1.7 nmol/mg protein [n = 2]) were essentially the same as that obtained in its presence (5′-nucleosidase = 612 ± 120 (n = 7) and sialic acid = 46.5 ± 5.5 [n = 2]). A determination for endoenzyme activity of the plasma membrane preparation in this case gyceraldehyde dehydrogenase [16], was also carried out in the presence and absence of Triton X-100. In the absence of this detergent, this enzyme activity was essentially negligible (2.55 ± 0.7 nmol/min/mg protein). In the presence of detergent, however, a substantial level of activity was observed (31.2 ± 5.2 nmol/min/mg protein). From these results it would appear that the vast majority of the vesicles are in the right-side-out orientation.

Discussion

From the results presented here we conclude that the L1210 cell vesicle preparations consist of two subpopulations, of which only one is competent for carrier-mediated transport of folate compounds. Both subpopulations of vesicles are accessible to substrate by simple diffusion alone. However, in the carrier-competent subpopulation, since equilibration is so rapid, the diffusion component of uptake is only apparent at high substrate concentrations or under conditions where carrier-mediated accumulation is suppressed. The data appear to exclude the possibility that the heterogeneity observed is related to "sideness" of the isolated membrane (right-side-out *vs.* inside-out).

The existence of different subpopulations of vesicles need not merely represent an artifact of the system but could reflect heterogeneity of the original intact cell population (e.g., resting *vs.* cycling cells). This hypothesis is difficult to support, however, since heterogeneity of transport by intact cells of the kind reported here has never been observed.

Even if differences between cells are negligible, it must be realized that the surface area of a typical vesicle is very small compared to the surface area of the whole cell. Thus transport-incompetent vesicles could arise simply because there are not enough carrier molecules per cell to partition among all of the vesicles. To see if this is plausible, we take a value of 0.2 μm for the radius of a typical vesicle [radius determined by electron microscopy, *see* ref. 23]. The surface area per vesicle is $A_v = 4\pi R^2 \simeq 0.5\,\mu m^2$. The radius of a typical L1210 cell is approximately 10 μm. After taking into account the usual factor of 3 for the percent of membrane incorporated into blebs and microvilli [17], the surface area per cell is approximately $A_c = 3 \times (4\pi R^2) \simeq 4{,}000\ \mu m^2$. This implies that a vesicle represents only 0.01% of a complete cell membrane. Clearly if the number of carrier molecules per cell is less than 10,000 then simple partitioning could ac-

count for the presence of transport-incompetent vesicles.

A final possibility which cannot be ignored is that carrier molecules are segregated into certain regions of the cell membrane and that only these regions give rise to transport-competent vesicles. In support of this notion are the well-known results of Tsan and Berlin [19, 20] who found that surface transport sites for both purines and amino acids were excluded from the parts of the membrane involved in endocytosis. It was subsequently shown that this exclusion from endocytic vesicles could be prevented by pretreatment of the cells with colchicine. In this connection it would be of interest to see if pretreatment with colchicine could affect the volume of the vesicle compartment incompetent for folate transport.

Finally, there is reason to believe from the results of our own studies and from other reports appearing in the literature that this type of functional heterogeneity is a property of isolated membrane preparations in general. We have derived similar evidence for compartmentation during studies[1] of [^{3}H]methotrexate transport in plasma membrane vesicles from Sarcoma 180 cells and human leukemia cells. Similar evidence has also been reported by others, for example, during measurements of amino acid uptake in plasma membrane vesicles prepared from 3T3 cells [4], uptake and efflux in plasma membrane vesicles from mouse fibroblasts [9], and uptake in rabbit renal brush border membrane vesicles [3, 8]. Since, in each of these reports, an explanation for this complexity was not sought, it can only be tentatively assumed that it relates to functional heterogeneity among subpopulations of vesicles. Although further study will be required to determine the generality and physical basis for these observations, our studies with folate compounds presented here could provide a basis for more adequate interpretation of transport data in this type of system.

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