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Membrane vesicles in the study of transport processes: a critical analysis of the experimental procedure

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A critical analysis of the use of membrane vesicles in the study of cotransport processes is presented. Transport experiments were simulated according to two different models, stressing those conditions that seemed more relevant in affecting the measurements. In particular, we observed that the experimental V_{max} values were underestimated. This underevaluation depended on the incubation time employed to measure the initial uptake rate and on the time necessary to wash the vesicles. Also the temperature and the composition of the washing solution, together with the Q_{10} of the transport process taken into consideration, had a consistent influence on the uptake. All the above mentioned effects were affected by the vesicle volume: the smaller the volume, the greater the understimate of the uptake. This theoretical analysis underlines, on the one side, that the experimental data should be interpreted with some caution, on the other, that the examined procedure allows an internal check of its validity by adopting suitable simulations of the experiments. The use of the presented models as a tool for the planning and the critical analysis of the experimental results is suggested.

Introduction

The aim of this work was to perform a theoretical analysis of an experimental method of measuring isotope fluxes across membrane vesicles. Two different mathematical models of carrier-mediated cotransport that seem very suitable, or at least extensively used, to describe transports in epithelial tissues, have been considered. Purified membrane vesicles have been largely used to describe transport phenomena, in particular in the study of secondary active transport depending on the presence of ion gradients. As it was pointed out by Heinz and Weinstein (1984) [1], this experimental approach has the advantage of avoiding the interference of metabolism, but it does not allow to study transport phenomena under steady-state conditions, since electrochemical potential gradients will dissipate towards equilibrium. As a consequence, only transient phenomena like the intravesicular accumulation of a cotransported solute, the so called overshoot, can be observed and theoretically simulated [1–4]. The solute uptake depends on many factors, such as the kinetic features of the transport, the experimental conditions, i.e. ions and solute gradients, and the properties of the membrane, as ion and water permeabilities [1,3]. However, we think that it is also important to take into account some effects that are a direct consequence of the used experimental techniques and that can significantly influence the values of the experimental measurements. In order to quantify these possible effects, we employed the theoretical transport models presented previously [1,3–5] and we simulated transport experiments stressing those conditions that seemed more relevant in affecting the measurements. In particular, the measure of the initial uptake rate, the effect of vesicle volumes and of vesicle washing, are the points that have been considered in this work.

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Glossary

a'_i, a''_i: activity of the solute (mM);
b', b'': activity of a monovalent driver cation (mM);
x', x'': amount of unloaded carrier (μmol · g^{-1});
X_T: total amount of carrier per g protein (μmol · g^{-1});
(bx)', (bx)'', (ax)', (ax)'': amount of binary complexes (μmol · g^{-1});

(abx)', (abx)'': amount of ternary complex (μmol · g^{-1});
P_D: diffusion coefficient of the solute (ml · g^{-1} · min^{-1});
P_+: cation permeability of the driver salt (ml · g^{-1} · min^{-1});
W: vesicle volume per g protein (ml · g^{-1});
P_0: rate coefficient of empty carrier translocation (min^{-1});
P_a: rate coefficient of binary complex (ax) for BTCM (min^{-1});
P_b: rate coefficient of binary complex (bx) for BTCM (min^{-1});
P_{ab}: rate coefficient of ternary complex (abx) for BTCM (min^{-1});
K: dissociation constant of the ternary complex for TCM (mM^2);
K_a: dissociation constant of the binary complex (ac) for BTCM (mM);
K_b: dissociation constant of the binary complex (bc) for BTCM (mM);
f_b: $(K_a \cdot K_b)/K_{ab}$
r: ratio of cation over anion permeability of the permeant salt;
α: ratio of rate coefficient of the translocation of the ternary complex over the unloaded carrier for TCM.

Materials and Methods

Brush border membrane vesicles (BBMV) from the midgut of mature lepidopteran larvae (*Philosamia cynthia*) and from rat jejunum were used.

Philosamia cynthia BBMV. BBMV were prepared from frozen midgut of fifth instar *Philosamia cynthia* larvae, by calcium precipitation as previously described [6]. The pellet from the second centrifugation step and the final pellet were resuspended in a medium containing 100 mM mannitol, 10 mM Hepes-Tris at pH 7.4. The final membrane pellet was resuspended at a protein concentration of 5–10 mg/ml as determined according to [7] with a Bio-Rad kit, using bovine serum albumine as a standard. BBMV were diluted with the same volume of a cocktail containing the labelled amino acid (approx. 80 μC/ml), 100 mM mannitol, 10 mM Hepes-Tris at pH 7.4. Transport experiments were performed in triplicate by a rapid filtration technique [6]. When leucine uptake values were measured as a function of external leucine concentration, isoosmolarity was maintained with mannitol. Short incubation times (from 0.2 to 7 s) were carried out with an automated apparatus (Innovativ Labor Ag CH-8134 Adiswil, Switzerland) consisting of a timer controlling both a shaker and an injector. 10 μl of BBMV suspension and 10 μl of labelled solution were placed on the bottom of a tube fitted into the shaker. At the start of the timer, the shaker was switched on and the two drops mixed. At the chosen incubation time, 1.8 ml of cold (5 °C) stop solution (150 mM NaCl, 1 mM Hepes-Tris at pH 7.4) were automatically injected into the test tube (first phase of washing). The sample was then filtered through a cellulose nitrate filter (0.65 μm pores) and rapidly rinsed with cold stop solution (second phase of washing). The mean value of the time elapsing from the stop solution injection to the filter insertion into the scintillaton liquid was 12 s. Radioactivity was then measured by a liquid scintillation spectrometer Tricarb Pakard model 300 C.

Rat BBMV. Rat BBMV were prepared from jejunum of albino male rats, Wistar strain, according to the procedure described by Esposito et al. 1985 [8]. The final pellet was resuspended in a medium containing 400 mM sorbitol, 10 mM Hepes-Tris at pH 7.4. The final membrane pellet was resuspended at a protein concetration of 2–4 mg/ml tested as reported above. L-Leucine uptake was measured by a rapid filtration technique. BBMV were diluted with the same volume of a cocktail containing the labelled amino acid (approx. 80 μC/ml), 10 mM Hepes-Tris, 200 mM sorbitol and 100 mM NaCl. The stop solution composition in this case was 125 mM NaCl, 150 mM sorbitol, 10 mM Hepes-Tris (pH 7.4). All other details follow those mentioned above for BBMV from *Philosamia cynthia*.

Kinetics were reported according to Eadie-Hofstee plot, where the intercept with the ordinate axis is the V_{max} value and the slope is $-K_m$ value.

Materials. L-[U-^{3}H]Leucine was obtained from the Radiochemical Centre (Amersham International, Amersham, U.K.). All other reagents were analytical grade products from BDH (Chemicals Ltd, Pole, U.K.).

Mathematical models

We adopted a cathegory of models previously illustrated in Ref. 3, and in particular two of them. The first one, that we call ternary complex model (TCM), has been shown in Ref. 1 and with some extensions and modifications in Ref. 3. The second one is more general, as it takes into account the transport of both binary and ternary complexes. It is called binary-ternary complex model (BTCM) and may be easily derived from the general theory [3] and the equations of unidirectional fluxes given by Heinz et al. (1972) [5].

For the TCM, that seems to be more extensively used in the literature, we employed the conventional set of parameters given by Heinz and Weinstein [1], except for $P_0 X_T$ and W (see Table I and Glossary; the denomination of parameters is given according to Andrietti et al. (1990) [3]); whereas the BTCM seems to be more suitably appliable to leucine transport found in *Philosamia cynthia* BBMV, where, even in the absence of any driver ion, leucine kinetics displays saturation, suggesting the presence of a carrier which can

cross the membrane also as a binary complex (carrier and leucine) [9]. In this case we preferred to use a set of parameters giving a reasonable fit to experimental data (Table I and Glossary). When different parameters were used, other than those reported in Table I, it is explicitly stated.

We think that these two models cover a wide range of possible applications and that the results derived from them may have a rather general range of validity.

In order to keep our theoretical elaboration comparable with the experimental measures, we considered the change in amount of tracer inside the vesicles (instead of instantaneous fluxes). Indeed, this is what was actually measured in the experiments.

More detailed aspects concerning the models, the meaning of parameters and the method of computation may be found in Refs. 1 and 5.

We present a number of simulations obtained from the models: in some cases theoretical curves have been fitted to experimental data. Whenever the experimental conditions were changed, still maintaining the previously fitted parameters, the curves are called predicted.

TABLE I

Standard values

Incubation solution: 25 °C; stop solution: 5 °C; washing time: 12 s (4+8 s).

	TCM	BTCM
P_D	0 ml·g^{-1}·min^{-1}	0 ml·g^{-1}·min^{-1}
P_+	2 ml·g^{-1}·min^{-1}	0.5 ml·g^{-1}·min^{-1}
P_0X_T	15 μmol·g^{-1}·min^{-1}	13.6 μmol·g^{-1}·min^{-1}
K	20 mM^2	–
K_a	–	1 mM
K_b	–	5 mM
f_b	–	1.4
W	3.5 ml·g^{-1}	3.5 ml·g^{-1}
P_a	–	36 min^{-1}
P_b	–	50 min^{-1}
P_{ab}	–	100 min^{-1}
a'	0.1–5 mM	0.1–5 mM
$a''(0)$	0 mM	0 mM
b'	100 mM	100 mM
$b''(0)$	0 mM	0 mM
r	1	1
α	1	–
Q_{10}	1.50 (diffusive processes)	1.50
Q_{10}	2.25 (cotransport processes)	2.25

Results

In Fig. 1 the experimental (2) and simulated (3) results of a leucine kinetics are presented. The uptakes were measured after 3 s of incubation, as a function of external leucine concentration (from 0.1 to 5 mM), in absence of the driver ion. The simulation was obtained by using the BTCM, which allows binary transport in absence of the driver cation b. The transport parameters obtained by fitting the simulated curves to experimental results are given in Table I. Note that in BTCM P_b, P_{ab}, f_b, K_b are not relevant when the driver b is absent ($b' = b'' = 0$). Predicted and experimental kinetics, obtained by measuring leucine uptake at 7 s, are also reported in Fig. 1 (curves 4 and 5). Note that the predicted curve was obtained by changing only the incubation time. The upper curve (1) of Fig. 1 corresponds to the simulated kinetics at 0 s.

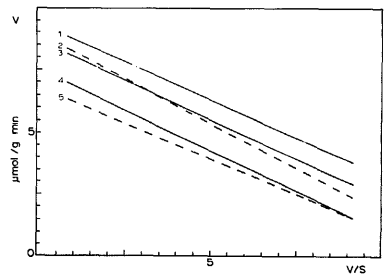

Fig. 1. Time effect on leucine initial rate kinetics in absence of driver ion; dotted lines correspond to experimental curves, continuous lines correspond to simulated curves according to BTCM: (2) kinetics at 3 s; (3) simulated curve at 3 s fitted to (2); (5) kinetics at 7 s; (4) predicted kinetics at 7 s; (1) simulated curve at 0 s.

The values of V_{max} and K_m corresponding to the curves of Fig. 1 are given in Table II.

For the TCM, we cannot give curves under the same conditions as those reported in Fig. 1, since no binary transport is allowed for such a model. Therefore, in Fig. 2 simulated kinetics according to TCM are shown, at 0, 6 s, 10 s, in presence of a driver gradient and with the parameter values given in Table I. In this figure also a potential difference, calculated according to

TABLE II

Theoretical and experimental kinetic parameters of leucine uptake (see Fig. 1)

Means ± S.E. Number of independent kinetics in parenthesis.

Initial time (s)	V_{max} (μmol·g^{-1}·min^{-1})	K_m (mM)
0	9.39	0.60
3 experimental	9.60 ± 0.8 (4)	0.74 ± 0.06
simulated	8.67	0.62
7 experimental	6.86 ± 0.74 (10)	0.59 ± 0.003
predicted	7.59	0.67

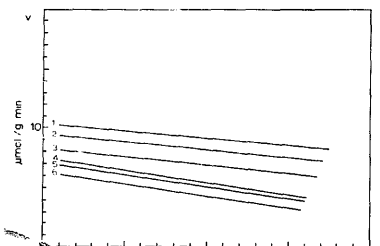

Fig. 2. Time effect on solute initial rate kinetics in presence of a driver ion gradient (100 mM outside the vesicles, 0 mM inside), simulated curves according to TCM, in presence (three upper curves) and in absence (three lower curves) of an electrical potential across vesicle membrane ($r = 0.2$: (1), (4) kinetics at 0 s; (2), (5) kinetics at 6 s; (3), (6) kinetics at 10 s.

Goldman-Hodgkin equation in case of a neutral carrier [1], has been taken into account (upper three curves). The electrical potential difference causes a consistent V_{max} increase.

When the driver ion is present also inside the vesicles, the retrodiffusion during the incubation time is obviously greatly enhanced, as shown in Fig. 3.

Fig. 4 shows how the vesicle volume W affects the kinetics of leucine uptake at 6 s. Note that the smaller the volume, the greater the deviation from the zero time kinetics.

The washing effect of the stop solution may be evaluated knowing that the vesicles were diluted about a hundred-fold in the stop solution during the first phase of the washing process (see Materials and Methods). This situation was simulated by starting with the concentration values a'' obtained from a previous calculation at a given time and making a new integration with a hundred-fold reduction of a'. In the second phase of the washing process (rapid filtration), no more external solute was present, and the last integration was performed accordingly. In both phases, the osmotic and ionic composition of the stop solution must be taken into account. Since the external solution was cold (5 °C), also the parameters regarding the solute transport changed accordingly. This was done by assuming a suitable Q_{10}, that was determined under two limit situations, i.e. those concerning physical or chemical processes. For a given set of parameters, that we presume to depend only on simple diffusion, we set an

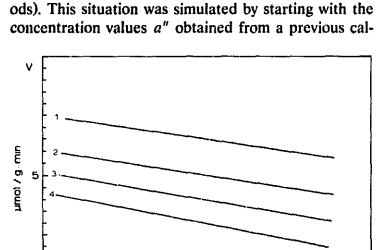

Fig. 3. Time effect on solute initial rate kinetics in presence of an equal amount of driver ion inside and outside the vesicles (100 mM) according to TCM: (1) kinetics at 0 s; (2) kinetics at 3 s; (3) kinetics at 6 s; (4) kinetics at 10 s.

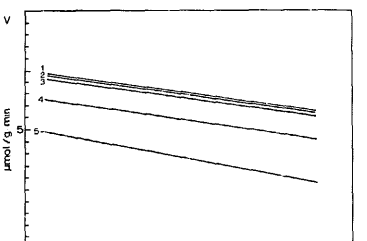

Fig. 4. Vesicle volume effect on solute kinetics simulated according to TCM at 6 s initial time: (1) reference kinetics at 0 s initial time; (2) $W = 5$ ml g^{-1}; (3) $W = 2.5$ ml g^{-1}; (4) $W = 1$ ml g^{-1}; (5) $W = 0.5$ ml g^{-1}.

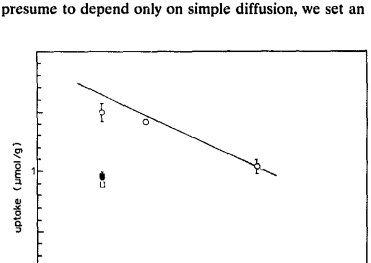

Fig. 5. Effect of washing time on leucine uptake at 1 min. Experimental values at different retrodiffusion times at 5 °C (○); fitted values of uptake at 5 °C (continuous line); experimental (■) and predicted value (□) at 25 °C. $Q_{10} = 2.25$, the parameter values are given in Table I except for $r = 0.5$, electrical potential calculated according to Goldman-Hodgkin model, neutral carrier, $a' = 0.5$ mM, $P_D = 1.5$ ml g^{-1} min^{-1}. The composition of the stop solution is given in Materials and Methods for rat BBMV. Other details in the text.

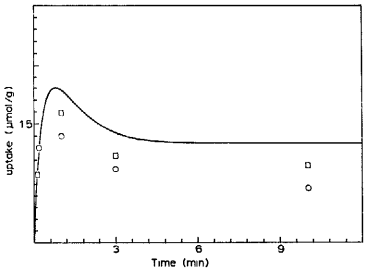

Fig. 6. Time course of leucine uptake in presence of a driver ion gradient (100 mM outside the vesicles; 0 mM inside): simulated uptake in the absence of washing (continuous line); predicted values with a 12 s washing time (4+8 s) and $Q_{10} = 2.25$ (□); experimental values of leucine uptake (○). The parameters are given in Table I, except for $r = 0.5$ (electrical potential calculated according to Goldman-Hodgkin model, neutral carrier), $P_D = 1.5$ ml g^{-1} min^{-1}, $a' = 0.5$ mM. The composition of the stop solution is reported in Materials and Methods for rat BBMV.

arbitrary $Q_{10} = 1.5$. For other parameters, involved in carrier mediated transport, we tried to give an evaluation based on the data reported in Fig. 5. In this experiment, the second phase of the washing process was constant at 8 s, while the first phase changed from 4 s to 32 s. In the abscissa of Fig. 5 the total washing time is reported. The experimental values of leucine uptake were reasonably fitted with a Q_{10} value of 2.25 (continuous line). Note that this Q_{10} value predicts a reduction of the uptake of about 45%, when washing occured at 25 °C, against the 37% of the experimental measure. Parameter values used in the fitting are reported in Table I, for TCM, except for $r = 0.5$ and $P_D = 1.5$ ml · g^{-1} · min^{-1} (the last value is based on unpublished experimental data). Note that these values also give a reasonable fitting to the experimental time course of leucine uptake, in BBMV from rat intestine, reported in Fig. 6.

Figs. 7 and 8 show how the kinetics change when the washing time effect is taken into account and no driver ion is present in the stop solution.

Fig. 7A shows leucine kinetics according to TCM in absence of washing, at 12 s (4 s + 8 s for the first and second phase of washing, respectively) and 36 s (12 s + 24 s) of washing time. Fig. 7B also shows washing time effect when the BTCM is used. The marked difference between the two figures depends obviously on the fact that in the TCM case no relevant retrodiffusion occurs, since the driver concentration inside the vesicles is very low at 3 s of incubation time. In the BTCM case, instead, the binary complex makes the main contribution to the retrodiffusion (carrier and amino acid). Note the deviation from linearity (Fig. 7B) when the washing time is higher. In other experimental conditions, for example when the driver ion is present on both sides of the vesicles, the washing time effects are more relevant, as it may be seen in Figs. 8A and B.

Fig. 9 shows the curves obtained under the same conditions of Fig. 8A, except for a Q_{10} value varying from 1.5 to 4 for the parameters involved in the carrier mediated transport. Observe that the effect of washing decreases with increasing values of Q_{10}.

Fig. 10 shows the effect of changing the vesicle volume W. Note that the smaller the volume, the greater the underestimate of the uptake, because of the outflux of the solute from the vesicles.

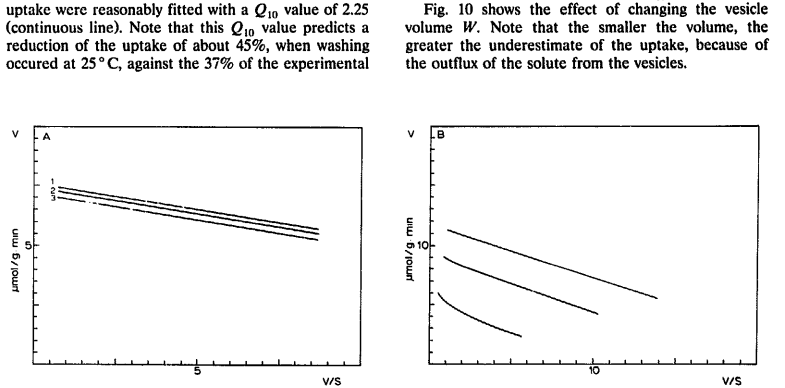

Fig. 7. Washing time effect on solute kinetics in presence of a driver gradient (100 mM outside the vesicles, 0 mM inside). (A) Simulation according to TCM: (1), (2), (3) 3 s initial time and 0, 12 s (4+8 s), 36 s (12+24 s) of washing time, respectively. (B) Simulation according to BTCM: (1), (2), (3) 3 s initial time and 0, 12 s (4+8 s), 36 s (12+24 s) of washing time, respectively. No driver ion in the stop solution. Other details in the text.

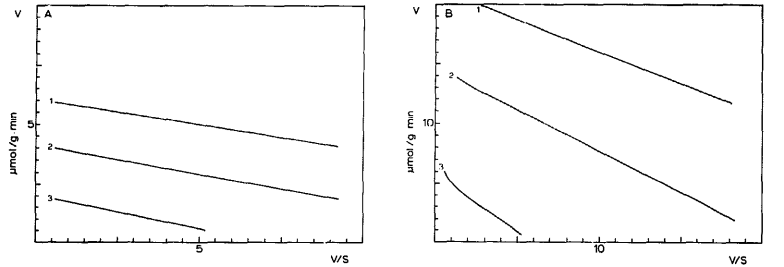

Fig. 8. Washing effect on solute kinetics in presence of an equal amount of driver inside and outside the vesicles (100 mM). (A) Simulation according to TCM: (1), (2), (3) 3 s initial time and 0, 12 s (4+8 s), 36 s (12+24 s) of washing time, respectively. (B) Simulation according to BTCM: (1), (2), (3) 3 s initial time and 0, 12 s (4+8 s), 36 s (12+24 s) of washing time, respectively. No driver ion in the stop solution. Other details in the text.

Figs. 11A and B show that the presence of the driver ion in the stop solution reduced the underestimate of the uptake in both the TCM and BTCM.

Discussion

In this work we have explored in some details the effect of the experimental procedure on the evaluation of kinetic parameters of cotransport V_{max} and K_m. For the correct determination of these values the measure of the initial uptake rate is required. Since the experimental initial uptake times are within a few seconds, it is worthwhile to evaluate the influence of the outflux from the vesicles during this incubation time. This problem has been considered by many authors, but in most cases it was approached from an experimental point of view [10,11]. As shown in Fig. 1 for BTCM and Figs. 2–4 for TCM, the time effect is relevant and it can be enhanced under particular experimental conditions, as in the presence of an electrical potential difference (Fig. 2), of a driver ion inside the vesicles (Fig. 3) or for small vesicle volumes (Fig. 4).

A second aspect of our analysis concerns the retrodiffusion occuring during vesicle washing . This is a well known phenomenon [10] that, as far as we know,

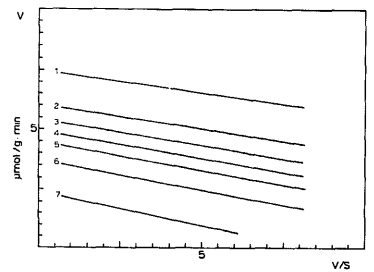

Fig. 9. Q_{10} variation effect on solute kinetics at 3 s initial time, in presence of an equal amount of driver ion inside and outside the vesicles (100 mM), according to TCM: (1) reference curve at 0 s initial time and 0 s of washing time; (2) 3 s initial time, 0 s washing time; (3)–(7) 3 s initial time, 12 s of washing time and 4, 3, 2.5, 2, 1.5 Q_{10} values, respectively, for parameters involving carrier-mediated processes. No driver ion is present in the stop solution. Other details in the text.

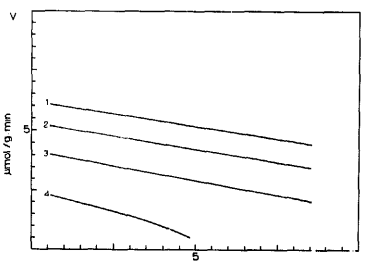

Fig. 10 Vesicle volume (W) variation effect on solute kinetics at 3 s initial time and 12 s of washing time in presence of an equal amount of driver ion inside and outside the vesicles (100 mM), according to TCM: (1) $W = 10$ ml g^{-1}; (2) $W = 5$ ml g^{-1}; (3) $W = 2.5$ ml g^{-1}; (4) $W = 1$ ml g^{-1}. No driver ion is present in the stop solution. Other details in the text.

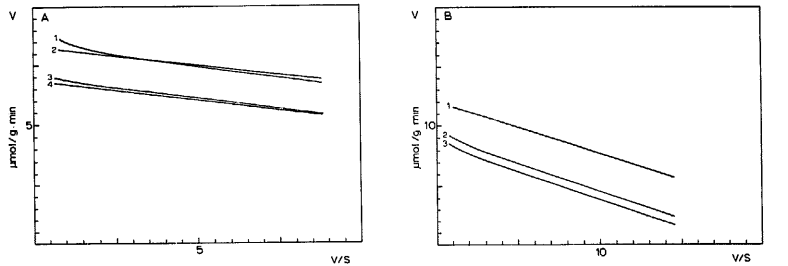

Fig. 11. Effect of the ionic stop solution composition on solute kinetics. (A) Simulation according to TCM, 12 s washing time: (1), 3 s initial time, stop solution containing 125 mM of driver ion, (2) 3 s initial time, no driver ion in the stop solution, (3) 10 s initial time, stop solution containing 125 mM of driver ion and (4) 10 s initial time and no driver concentration in the stop solution. (B) Simulation according to BTCM, 3 s initial time: (1), no washing, (2) 12 s washing time, stop solution containing 125 mM of driver ion and (3), 12 s washing time, no driver ion concentration in the stop solution. $r = 0.5$, electrical potential calculated according to Goldman-Hodgkin model, neutral carrier.

has never been analyzed quantitatively. This effect may vary according to the different experimental conditions. Both outfluxes, occurring during the incubation and the washing, may cause a relevant reduction of the uptake values and consequently of V_{max}, but this effect depends on the features of the transport model considered and on the experimental conditions. It is much less relevant in TCM than in BTCM when the driver is present only outside the vesicles (Figs. 7A and B). Moreover, retrodiffusion due to vesicle washing is reduced by the low temperature of the stop solution and it is a decreasing function of the Q_{10} of the carrier mediated processes (Fig. 9).

Moreover, retrodiffusion effects are greatly enhanced by low vesicle volumes (Fig. 10) and are influenced by the composition of the stop solution (Figs. 11A and B). In fact, the presence of the driver ion in the stop solution reduces the amount of the retrodiffusion due to the washing procedure. The slight deviation from linearity of the kinetics is due to the fact that even a hundred-fold dilution with the stop solution leaves an external solute, mostly at a higher concentration, sufficient to reduce the retrodiffusion. This deviation from the linearity of the Eadie-Hofstee plot, usually explained by the presence of a diffusion component and/or an additional carrier mediated transport system, might be attributed to the effect of the washing procedure. In this case the effect should be eliminated by adequately changing the stop solution composition.

We did not consider the effect of water flux across the vesicle membranes. In fact, when the stop solution is isotonic with the initial solution inside the vesicles, a slight osmotic gradient may be created during incubation or washing time. However, the effect of the induced water flux, with values of water permeability given in the literature [12], does not significantly influence the kinetics. Instead, in time course uptake experiments, more dramatic changes induced by water movement can be observed [3].

We would underline that V_{max} variations observed under different experimental conditions should be regarded with some caution, whereas K_m values seem to be more independent from the experimental methods used. On the other hand, a critical analysis, as the one presented here, is possible only when the process considered is 'simple' as in BBMV, so that transport phenomena may be mathematically described and it is therefore possible to evaluate the influence of the experimental procedures on the kinetic features of the transport model considered.

In this present work we have tried to examine some of the most common experimental situations. Many other cases could, of course, be taken into account. However, apart from some obvious general indications, i.e. to use very short incubation times, to wash the vesicles rapidly and with a cold stop solution, to check the vesicle volumes and to avoid any initial osmotic gradient, it is not easy to give specific recommendations, because the error due to the experimental procedure has a different weight depending on the transport model and the particular conditions of the experiment. For this reason we suggest that the models of transport presented here, apart from the problem of their intrinsic validity, may be used as a heuristic tool to program experiments and for a better understanding of the results.

The authors will be happy to provide software facilities to whosoever may be interested.

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