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Na^+-Gradient-Stimulated AIB Transport in Membrane Vesicles from Ehrlich Ascites Cells

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Summary. Plasma membrane vesicles derived from Ehrlich ascites cells can accumulate 2-aminoisobutyric acid (AIB) twofold, in the absence of ion gradients or potential differences. In addition, AIB uptake is stimulated specifically by the presence of a Na^+ chemical gradient (high Na^+ outside). The nature of the counterion (e.g., K^+, Li^+, Cs^+, or ethanolamine) inside the vesicle does not affect the qualitative response although quantitative differences are observed. The level of AIB present in the vesicle decreases as the Na^+-gradient is dissipated. Gramicidin, which increases the rate of ion gradient dissipation, inhibits the gradient-stimulated uptake. Valinomycin stimulates AIB uptake when Na^+ is present outside the vesicles and K^+ is inside, probably by producing a diffusion potential which increases the electrochemical potential difference for Na^+. As the Na^+-gradient dissipates, AIB accumulation exceeds that predicted from 100% transfer of the energy from the Na^+ chemical gradient if a 1:1 relationship between amino acid and Na^+ coupling exists. It is possible that a diffusion potential adds to the chemical gradient for Na^+ making the electrochemical potential difference for Na^+ adequate to energize AIB accumulation. Ouabain inhibits gradient-stimulated AIB uptake without measurable effects on the ion distributions, thus showing a direct action of ouabain on amino acid transport.

The active transport of organic molecules in eukaryotic cells is frequently associated with the requirement for Na^+ ions at the external surface of the membrane. The search for an energy source for this Na^+-dependent transport has led to the formulation of several theories on the subject. The most widely accepted of these is the Na^+-gradient hypothesis (Crane, Miller & Bihler, 1961; Crane, 1965; Schultz & Curran, 1970) which states that the Na^+-electrochemical gradient that exists across the cell membrane drives the uptake of organic molecules, the Na^+ flux being coupled to the flux of the organic molecules into the cell. In a number of reports (Riggs, Walker & Christensen, 1958; Eddy, Mulcahy & Thompson, 1967; Eddy, 1968; Jacquez & Schafer, 1969; Potashner & Johnstone, 1970, 1971; Lin & Johnstone, 1971; Reid & Eddy, 1971), it was concluded that under certain

conditions the chemical gradient for Na^+ did not possess sufficient energy to drive the transport of the solute, and a contribution to the driving force from the K^+-gradient has also been considered (Reid & Eddy, 1971; Schafer & Heinz, 1971). However, other investigations (Lin & Johnstone, 1971; Schafer & Heinz, 1971; Johnstone, 1972, 1974; Kimmich, 1973; Kimmich & Randles, 1973*a*, *b*; Tucker & Kimmich, 1973) suggest that even when the latter energy source is considered, the energy supply from ion gradients may be insufficient and a real question exists whether other sources of energy are used under physiological conditions.

One particularly compelling line of evidence consistent with the Na^+-gradient hypothesis is that ouabain, a cardiac glycoside which specifically inhibits Na^+ transport by inhibiting the $Na^+ + K^+$-ATPase, interferes with the transport of organic molecules, presumably because it brings about the dissipation of the ion gradients. In fact, it has been noted that in preparations of the small intestine (Chez, Palmer, Schultz & Curran, 1967), ouabain is much less effective as an inhibitor of solute flow if a Na^+-gradient is maintained. However, this interpretation is not necessarily correct since in pancreas (Lin & Johnstone, 1971) and in Ehrlich cells (Bittner & Heinz, 1963) evidence for a direct action of ouabain on the transport of organic molecules has also been presented.

We have recently developed (Colombini & Johnstone, 1974) a plasma membrane system to study Na^+-dependent amino acid transport at the subcellular level. We have obtained evidence that amino acids may be accumulated in these vesicles against their chemical gradients in the absence of an electrochemical potential difference for Na^+. In this report we present further evidence to support this conclusion and, in addition, show that ion gradients, or more particularly a Na^+-gradient, does increase amino acid transport and accumulation. The inhibition by ouabain of amino acid transport in the absence of measurable effects on cation distributions is presented as evidence for a direct action of ouabain on the amino acid transport system.

Materials and Methods

Plasma membranes were obtained from Ehrlich ascites cells as described previously (Colombini & Johnstone, 1973, 1974). The method for measuring uptake into and efflux out of the plasma membrane vesicles has also been described (Colombini & Johnstone, 1974).

Uptake Measurements in the Presence of Ion Gradients

The vesicles (5 to 10 mg protein/ml medium) were preincubated with 100 mM of a given neutral salt for 30 min at 37 °C in a medium which also contained 0.1 mM $MgCl_2$,

0.1 mM $CaCl_2$ and 5 mM Tris HCl, pH 7.5. Either Li^+, Na^+, K^+, Rb^+ or Cs^+ (as chloride salts) were used. After this preincubation the vesicles were centrifuged at $12{,}000 \times g$ for 3.5 min in an Eppendorf Micro Centrifuge using 1.5-ml micro test tubes. The supernatant was discarded and the pellet was resuspended in a fresh medium similar to the preincubation medium except that the alkali metal cation in the solution was replaced with another such cation. Also present in the medium was the radioactive substrate whose uptake was being studied. Thus, transport measurements were started immediately upon switching from one cation to another to take advantage of the ion gradients. Incubations to measure uptake were at 20 °C unless otherwise stated. Samples were taken at the times shown in the figures. The samples were diluted, filtered and washed with ice cold Na^+ medium as described previously (Colombini & Johnstone, 1974). When movement of $^{86}Rb^+$ was assayed, the dilution medium consisted of 1.7 ml Na^+ medium and 0.2 ml of 100 mM RbCl in 5 mM Tris Cl, pH 7.5.

Transfer of a solute across a membrane may be expressed as μmoles transferred per cm^2 membrane or per unit amount of protein if the surface is unknown and it is assumed that the surface area is proportional to the protein content. Since the concentration of the solute in the originating fluid is known, the rate of transfer of solute can also be expressed as μliter equivalents of medium cleared of solute per mg protein. This expression has the advantage that the flux of solutes present at different concentrations may be compared directly and we have chosen this form to express our data.

Protein was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as standard. All radioactive samples were counted using a modified Bray's solution (Bray, 1960). The membranes were filtered on glass fiber filters, the whole filter being placed in a liquid scintillation vial and counted. The filter had no detectable effect on the counting efficiency or on the spillage of counts from the $^{22}Na^+$ or $^{86}Rb^+$ channel into the ^{14}C channel. The extent of spillage into the carbon channel was about 30% for $^{22}Na^+$ and about 10% for $^{86}Rb^+$.

All radioactive chemicals were purchased from New England Nuclear, Boston, Mass., while nonradioactive compounds were purchased from either Sigma Chemical Co., St. Louis, Mo., or Fisher Scientific Co., Montreal, Canada.

Results

AIB Accumulation in the Vesicles

In our previous paper (Colombini & Johnstone, 1974) we presented evidence that 2-aminoisobutyric acid (AIB) was transported into the vesicles. Indirect evidence indicated that an accumulation against a concentration gradient was taking place. For example, the steady-state level of AIB associated with the vesicles did not increase proportionally with the medium AIB concentration as would be expected if the system had attained an equilibrium position. We also observed that AIB influx into the vesicles was faster than efflux. Although some equilibrating systems may show unequal fluxes under certain conditions, the data may also be consistent with an intravesicular accumulation of AIB. To test directly for an accumulation against a concentration gradient, it is necessary to measure the intravesicular volume. The combined use of $^3H^1HO$ (to measure the total

space) and ^{14}C dextran (an extravesicular space marker) followed by centrifugation led to large apparent intravesicular volumes. If ^{14}C-sucrose instead of ^{14}C-dextran was used, the apparent intravesicular space was much smaller. However, the ^{14}C-sucrose-impermeable space measured in this way was so small compared to the $^{3}H^{1}HO$ space that the estimation of intravesicular space was subject to large errors. The reason for the apparent difference in intravesicular volume obtained with ^{14}C-dextran and ^{14}C-sucrose is not known. However, it is conceivable that not all the vesicles are sealed to relatively small molecules like sucrose but are sealed to high molecular weight dextran (60 to 90×10^3 Daltons). We therefore changed our approach and tried to estimate the intravesicular space by using intravesicular space markers. Ideally, these markers should be substances which distribute themselves equally between the vesicular water and the medium but are not rapidly washed out of the vesicle at 0 °C. Thus, by applying the same technique to these substances as to the transported solute under investigation one should obtain a measure of the intravesicular volume. We chose 3-O-methylglucose as a possible intravesicular marker. In the intact cell it is transported by a facilitated process but not accumulated (Crane, Field & Cori, 1957). In our hands, these data were confirmed and further, no Na^+-dependence was observed and a steady state was attained quickly. In the vesicle also, the uptake of ^{14}C-3-O-methylglucose is very rapid (Fig. 1) usually attaining steady state by 1 to 5 min of incubation. To ascertain that loss from the vesicles at 0 °C is considerably less rapid than uptake at 20 °C, efflux of 3-O-methylglucose was measured at 0 °C. Although the rate of loss is significant, it is evident from the curve in Fig. 1 that in 5 or 10 sec, the time required for separation and filtration of the membranes, the loss would be negligible. Hence, 3-O-methylglucose was used as a marker for intravesicular volume. $^{22}Na^+$ distribution in the standard Na^+ medium was also used to estimate intravesicular volume. The uptake of $^{22}Na^+$ reaches steady state at 30 min. The distribution of $^{22}Na^+$ in the vesicles at steady state is, within experimental error, equal to the 3-O-methylglucose distribution. That is, at steady state the μliters of medium cleared of solute are equal for $^{22}Na^+$ and ^{14}C-3-O-methylglucose. Similar values for the volume of medium cleared of solute are obtained with $^{86}Rb^+$ in a medium containing 5 mM $^{86}Rb^+$ and 100 mM NaCl and also with $^{36}Cl^-$ in 100 mM NaCl. These data suggest that in these experiments, the markers attain an equilibrium distribution with the same concentration of solute being present on both sides of the membrane and that there is insufficient potential difference across the vesicular membrane or insufficient nonpermeable ionic material trapped in the vesicles to cause an asymmetric

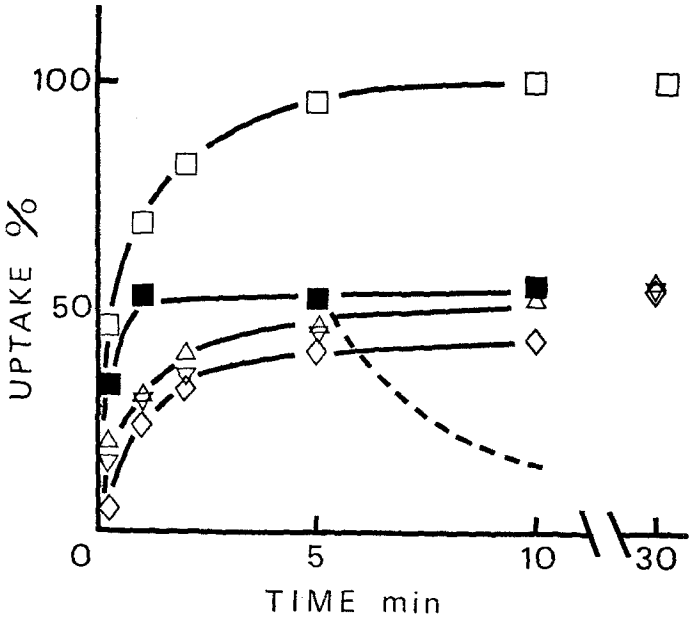

Fig. 1. Comparison of AIB uptake with the uptake of other solutes. Uptakes of all solutes are expressed as a percentage of AIB at 10 min. AIB uptake at 10 min varied between 0.26 and 0.3 μliter of medium cleared of substrate/mg protein. (S.A.=specific activity, cpm/nmole). [1-^{14}C]AIB, 0.4 mM, S.A. 22×10^3 (□); 3-O-methyl-^{14}C-D-glucose, 1.0 mM, S. A. 5.3×10^3 (■); $^{22}Na^+$, 100 mM, S.A. 40 (△); $^{36}Cl^-$, 100 mM, S.A. 78 (▽); $^{86}Rb^+$, 5.0 mM, S.A. 950 (◇). All uptake measurements were made at 20 °C in a Na^+ medium with vesicles preincubated as described (Colombini & Johnstone, 1974). The dotted line shows the efflux of 3-O-methylglucose at 0 °C

distribution of the positively and negatively charged particles. It should be noted in this context that the volume of medium cleared of AIB at steady state exceeds by a factor of two that obtained with the four markers.

Effect of Asymmetric Cation Distributions on AIB Uptake

The Na^+-gradient hypothesis states that amino acid transport may be energized by energy available from the Na^+ electrochemical potential difference (Crane, 1965; Schultz & Curran, 1970; Terry & Vidaver, 1973). A contribution of energy from the K^+-gradient to organic solute accumulation has also been proposed (Reid & Eddy, 1971; Schafer & Heinz, 1971). Hence, we decided to examine whether asymmetric distributions of cations would affect the transport activity of AIB in these vesicles. Our previous

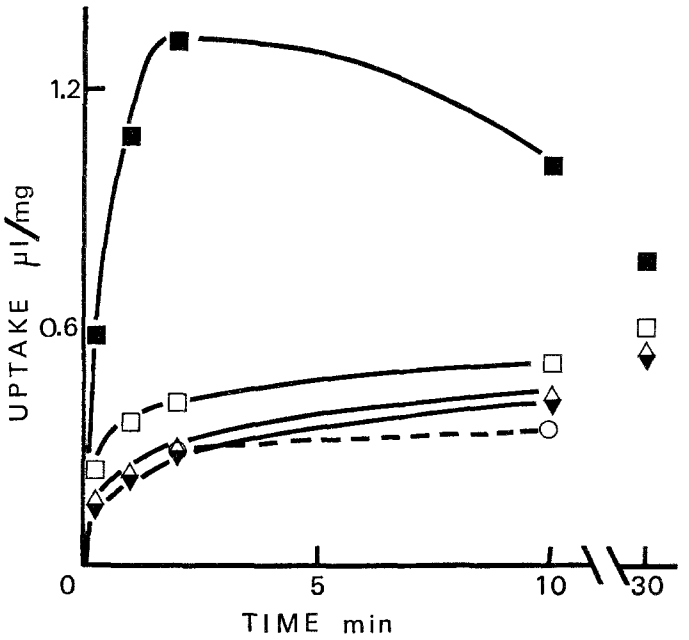

Fig. 2. $Na^+ + K^+$-gradient-stimulated AIB, and 3-O-methylglucose uptake. Vesicles were preincubated with 100 mM KCl or NaCl and then transferred to a medium containing either 100 mM NaCl or 100 mM KCl in addition to the ^{14}C-labeled solute (AIB or 3-O-methylglucose). [1-^{14}C]AIB (■, □, △, ▼) and 3-O-methyl-^{14}C-glucose (○) transport; Na_o^+ and K_i^+ (■, ○); Na_o^+ and Na_i^+ (□); K_o^+ and K_i^+ (△); Na_i^+ and K_o^+ (▼). The subscripts $_o$ and $_i$ refer to extra- and intravesicular, respectively. Concentration and specific activities of ^{14}C compounds used are as given in Fig. 1

studies (Colombini & Johnstone, 1974; *see also* Fig. 1) showed that an asymmetric Na^+ distribution is not required to obtain a twofold AIB accumulation. The data in Fig. 2 demonstrate that when vesicles are preincubated in a K^+ medium and then transferred to a Na^+ medium, a dramatic stimulation in AIB uptake occurs, reaching a maximum at 1 to 2 min after transfer to the Na^+ medium. Thereafter, the AIB level decreases approaching a steady-state level which is the same as that reached in vesicles pre-equilibrated in a Na^+ medium. This decrement in ^{14}C-AIB correlates rather well with the decreasing asymmetries of Na^+ and K^+ as measured by $^{86}Rb^+$ exit and $^{22}Na^+$ entry (Fig. 3). When ion gradients were allowed to dissipate for 30 min before the addition of AIB to the medium, AIB uptake was essentially equal to that in the control Na^+ medium. These data show that the presence of the gradient(s) is necessary to achieve the increase in AIB uptake.

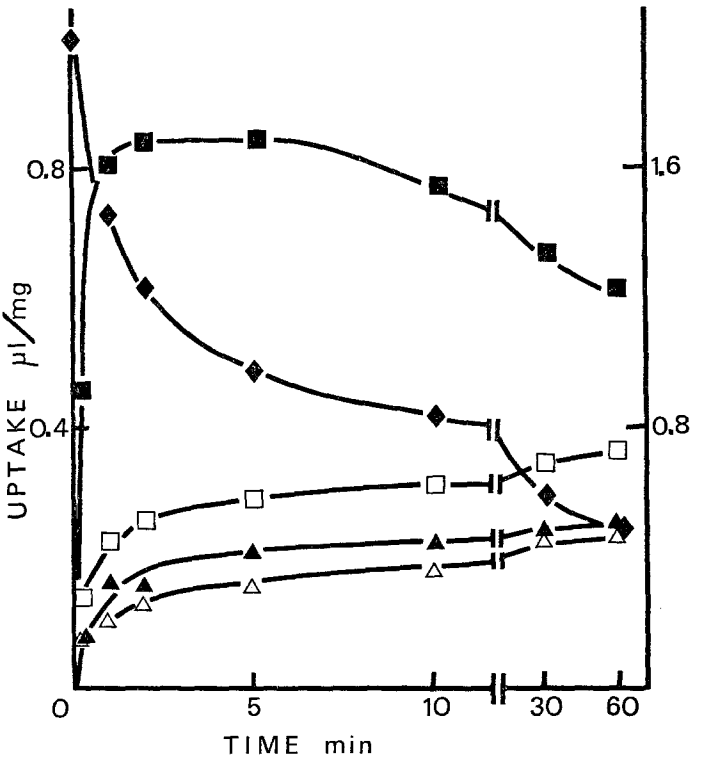

Fig. 3. Correlation of [1-^{14}C]AIB transport with $^{22}Na^+$ and $^{86}Rb^+$ fluxes. AIB uptake (■, □); ^{22}Na uptake (▲, △); $^{86}Rb^+$ efflux (◆). Hollow symbols represent the situation with Na^+ on both sides of the vesicle, and the filled symbols with intravesicular K^+ (or Rb^+) and extravesicular Na^+. The scale on the ordinate at right is doubled for the $^{86}Rb^+$ efflux. Concentrations and specific activities were as given in Fig. 1

If the Na^+-K^+ asymmetry is reversed (Fig. 2), i.e. if the vesicles are preincubated in Na^+ and switched to a K^+ medium, there is no stimulation of AIB uptake, indeed there may be a small inhibition in AIB uptake compared to the control level. Therefore, only normally directed Na^+- and K^+-gradients were able to stimulate AIB uptake.

It should be noted that the effect of the asymmetric distribution of cations on the rate of AIB uptake is not paralleled by a proportionate increase in the rate of Na^+ uptake, although it is evident from Fig. 3 that $^{22}Na^+$ uptake is somewhat increased upon imposition of gradients. The increase in the rate of Na^+ uptake that is observed in the presence of Na^+- and K^+-gradients may be partly due to a slight swelling of the vesicles at early times since a similar increase in uptake is also observed with 3-O-methylglucose.

Cation Specificity for Gradient-Stimulated Transport

The question arises whether one or both ion gradients are required and whether other cation gradients can mimic the action of $Na^+ + K^+$. To test for cation specificity, Na^+ was replaced by Li^+ or Cs^+ (Fig. 4). The data show that the absence of external Na^+ results in a greatly diminished uptake of AIB irrespective of the combination of cations used. Internal K^+ is not essential. So long as extravesicular Na^+ is present, AIB uptake is similar with intravesicular K^+, Li^+ or Cs^+. Hence, the Na^+-gradient is the major factor stimulating AIB uptake. The differences seen in the rates of uptake of AIB with intravesicular Li^+, K^+ or Cs^+ seem to be due to differences in the permeabilities of these ions. Thus, the rate of $^{22}Na^+$ entry also increases as the counterion, that is the internal ion, is changed in the sequence $Cs^+ > K^+ > Li^+$, correlating with the order of uptake of AIB, i.e. $Cs^+ > K^+ > Li^+$. Not only is uptake of AIB increased, but efflux is also greater in Cs^+ than in Li^+. That the nature of the counterion to Na^+ is not critical is borne out by the observation that ethanolamine can replace the intravesicular cation. Although the rate of AIB uptake is lower in ethanolamine than with the alkali metal ions, there is nonetheless a marked stimulation of AIB uptake (Fig. 5).

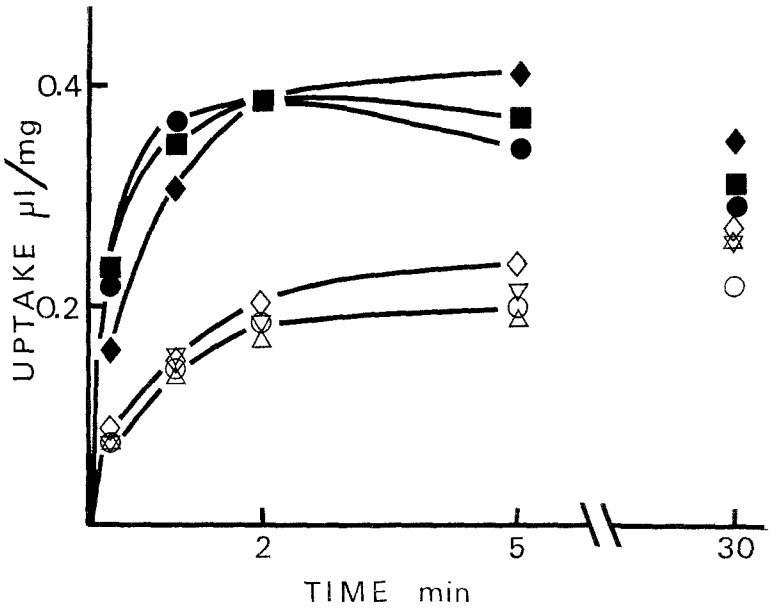

Fig. 4. The role of the counterion in gradient-stimulated AIB transport. [1-^{14}C]AIB uptake with K_i^+, Na_o^+ (■); Cs_i^+, Na_o^+ (●); Li_i^+, Na_o^+ (◆); K_i^+, Li_o^+ (◇); Cs_i^+, Li_o^+ (▽); K_i^+, Cs_o^+ (○); Li_i^+, Cs_o^+ (△). Specific activity and concentration of AIB were as in Fig.1

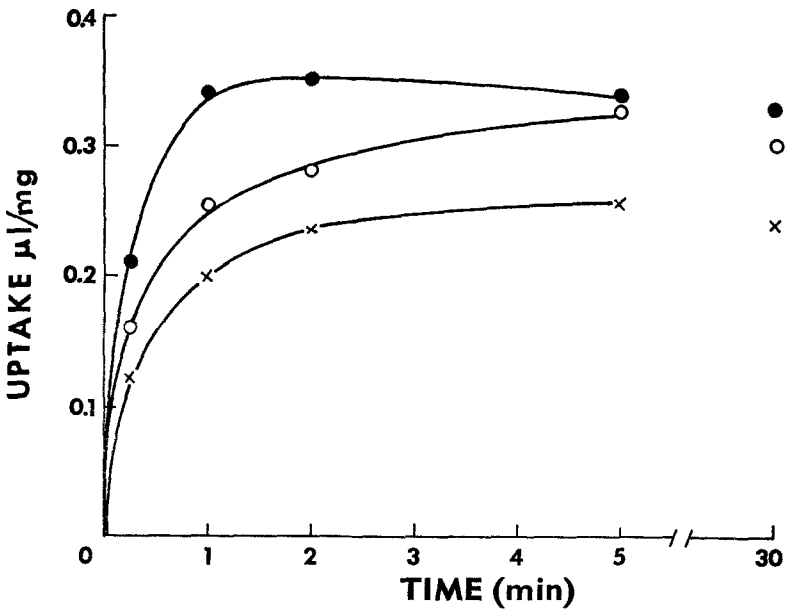

Fig. 5. Transport of [1-^{14}C]AIB with ethanolamine as counterion. Na^+ was present on the outside throughout, and Na^+ (×), K^+ (•) or ethanolamine (○) were present inside the vesicles. The specific activity and concentration of AIB were the same as in Fig. 1

Energetics of the Gradient-Stimulated AIB Uptake

If the cation gradients are exerting their effect on AIB accumulation by providing energy for the process, the energy inherent in the cation gradients should at all times exceed the energy required for increased AIB accumulation. If AIB accumulation is affected in some other way by the gradients (one possibility being carrier orientation while the energy for the transport process comes from sources other than the ion gradients), increased AIB accumulation may persist for a time even when the ion gradients are dissipated. Since the Na^+-gradient appears to be the only gradient coupled to AIB translocation, we followed the rate of decay of the Na^+-gradient to determine if, at all times, the free energy in the Na^+-gradient would exceed the increase in free energy required for AIB accumulation. Assuming (a) there is no potential difference across the membrane, (b) that the activity coefficients in the vesicles are the same as in the medium, and (c) the coupling between AIB and Na^+ is 1:1, we reasoned as follows:

Maximal energy available per mole of Na^+ from the Na^+-gradient

$$= RT \ln \frac{[Na_o^+]}{[Na_i^+]}$$

Minimal energy required per mole for AIB taken up

$$= RT \ln \frac{[\mathrm{AIB}_i]_G}{[\mathrm{AIB}_o]}$$

Energy available for base-line accumulation (in absence of ion gradients)

$$= RT \ln \frac{[\mathrm{AIB}_i]_{\mathrm{Na}}}{[\mathrm{AIB}_o]}$$

where the subscripts o and i refer to outside and inside the vesicle, respectively, and the subscripts G and Na refer to the intravesicular AIB concentration in the presence of $Na^+ + K^+$-gradients and in Na^+ medium in the absence of gradients, respectively. The energy available in the vesicle from other sources is expressed as the ability to accumulate AIB in the absence of gradients. If the Na^+-gradient provides sufficient energy to account for the stimulated AIB uptake:

$$RT \ln \frac{[\mathrm{Na}_o^+]}{[\mathrm{Na}_i^+]} + RT \ln \frac{[\mathrm{AIB}_i]_{\mathrm{Na}}}{[\mathrm{AIB}_o]} \geqq RT \ln \frac{[\mathrm{AIB}_i]_G}{[\mathrm{AIB}_o]} \tag{1}$$

$$\therefore \frac{[\mathrm{Na}_o^+][\mathrm{AIB}_i]_{\mathrm{Na}}}{[\mathrm{Na}_i^+][\mathrm{AIB}_o]} \geqq \frac{[\mathrm{AIB}_i]_G}{[\mathrm{AIB}_o]} \tag{2}$$

$$\frac{[\mathrm{Na}_0^+]}{[\mathrm{Na}_i^+]} \geqq \frac{[\mathrm{AIB}_i]_G}{[\mathrm{AIB}_i]_{\mathrm{Na}}} \quad \text{where } \frac{[\mathrm{AIB}_i]_G}{[\mathrm{AIB}_i]_{\mathrm{Na}}} = \text{fractional increase in } [\mathrm{AIB}_i] \tag{3}$$

but

$$\frac{[\mathrm{Na}_o^+]}{[\mathrm{Na}_i^+]} = \frac{[\mathrm{Na}_o^+](\mathrm{K}^+ \text{ moles}_{(i)} + \mathrm{Na}^+ \text{ moles}_{(i)})}{0.1\ \mathrm{Na}^+ \text{ moles}_{(i)}} \quad (\textit{see} \text{ Appendix}) \tag{4}$$

where K^+ moles$_{(i)}$ and Na^+ moles$_{(i)}$ are the amounts of Na^+ and K^+ in the vesicles expressed in moles, and the concentrations are in molar quantities. Therefore, $\frac{[\mathrm{Na}_o^+]}{[\mathrm{Na}_i^+]}$ is expressed in terms of measurable quantities without having to assume a value for the vesicular space. If sufficient energy is available from the Na^+ chemical gradient then

$$\underbrace{\frac{[\mathrm{Na}_o^+]}{[\mathrm{Na}_i^+]}}_{(A)} - \underbrace{\frac{[\mathrm{AIB}]_G}{[\mathrm{AIB}]_{\mathrm{Na}}}}_{(B)} \geqq 0. \tag{5}$$

The difference was determined at all times during gradient-stimulated uptake (Fig. 6). The results from four different membrane preparations with

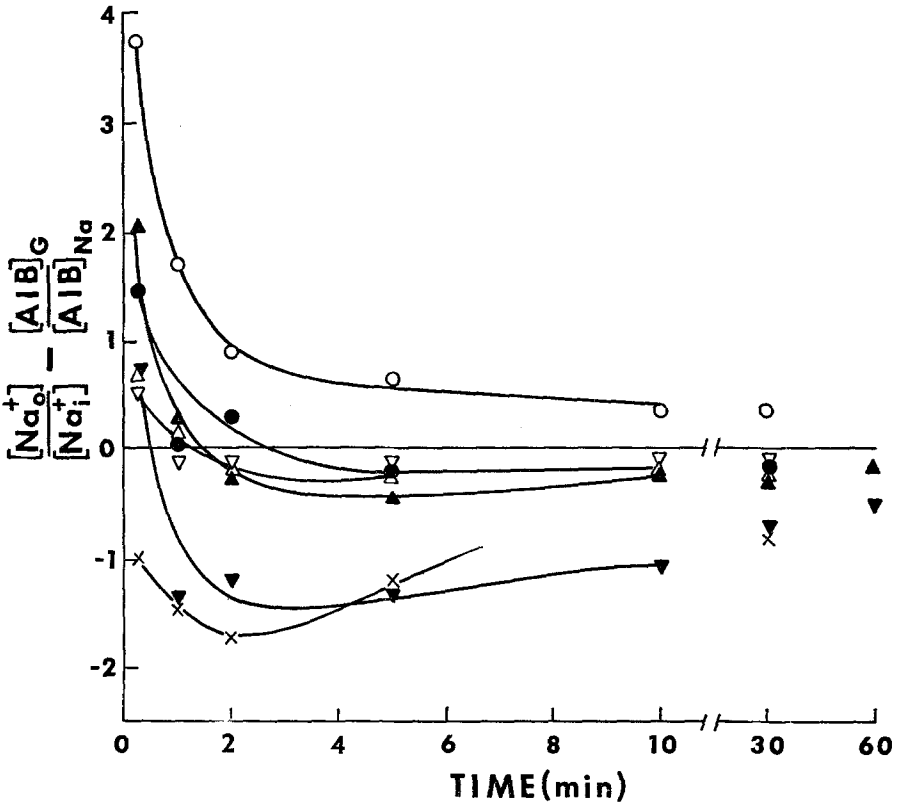

Fig. 6. Energetics of Na^+-gradient-stimulated transport of [1-^{14}C]AIB. Na^+ was present extravesicularly throughout. K^+ (▽, △, ▼, ▲, ×), Li^+ (●) or ethanolamine (○) were present inside the vesicles. The curve (×) represents the same membrane preparation as in curve (▲), except that 5.0 μg/ml of valinomycin was present in (×). The final ethanol concentration in (×) was 0.1%. Negative values indicate that insufficient energy is available from the Na^+ chemical gradient to explain gradient-stimulated transport. The specific activity and concentration of AIB were as indicated in Fig. 1

K^+ as counterion are shown in addition to data with other counterions using other membrane preparations. It is apparent from Fig. 3 that in a general way, AIB accumulation decreases as the Na^+-gradient decreases. However, the data in Fig. 6 show that after two minutes of incubation the difference between terms A and B is negative with K^+ as counterion, almost zero with Li^+ as counterion and positive with ethanolamine. If valinomycin is present in addition to K^+, the difference is more negative. The experimental data have shown that the numerical value of term A in Eq. (5) changes less when the counterion is changed from K^+ to Li^+ to ethanolamine than does the numerical value of term B in Eq. (5) (for example, *see* Figs. 3, 4 and 5). Therefore, in the data plotted in Fig. 6 according to

Eq. (5), the numerical value of term B determines to a large extent whether the difference is positive or negative. These data suggest two possibilities: (1) The transport system is activated to different extents by different counterions. This possibility seems unlikely since a cation like ethanolamine is unlikely to be an alternate activator to an alkali metal cation. (2) The different counterions set up diffusion potentials which are proportional to the rates of diffusion of the respective counterions with $K^+ > Li^+ >$ ethanolamine. Therefore, to the chemical potential difference for Na^+ one needs to add the electrical potential difference to obtain the true value for the energy available for AIB accumulation. Where the diffusion potential is small as with Li^+, AIB accumulation approaches the value predicted from the Na^+ chemical potential difference. When the diffusion potential is increased with valinomycin, accumulation of AIB exceeds significantly the value predicted from the Na^+ chemical potential difference. With ethanolamine, which might be expected to migrate less rapidly than Na^+, the diffusion potential is probably reversed with the inside of the vesicle becoming positive. Therefore, AIB accumulation is less than predicted from the chemical potential difference and the difference between terms A and B in Eq. (5) becomes positive. Computations show that the magnitude of the diffusion potential across the vesicle membrane would have to be between 5 and 20 mV (inside negative) with the different cations if the energy requirements for AIB uptake are to be met from the electrochemical potential difference for Na^+. If this interpretation of potential differences is found to be incorrect, one would have to postulate that another energy source is available in the vesicle and is used for AIB accumulation and that the efficiency of using the energy source depends on the nature of the counterion.

The pH Dependence of Gradient-Stimulated AIB Transport

The effect of pH on gradient-stimulated AIB transport was examined using the same buffer system throughout (bis tris propane-hepes, adjusted to the required pH with NaOH or HCl) with the pH varying from 5.5 to 9.0. All preincubations in K^+ were carried out at pH 7.5 to avoid long exposures to acidic or alkaline media. Thus, only the external pH was varied directly. After preincubation, the vesicles were transferred to a Na^+ medium of the appropriate pH and AIB uptake was followed (Fig. 7). For clarification, the data were replotted as a contour map (Fig. 8) using the shape of the curves in Fig. 7 to determine the position of the lines of equal uptake. The peak of maximum uptake occurs after 1 to 2 min at pH 7.5. Two aspects of the effect of pH are worthy of note. (1) The rate of uptake increases with

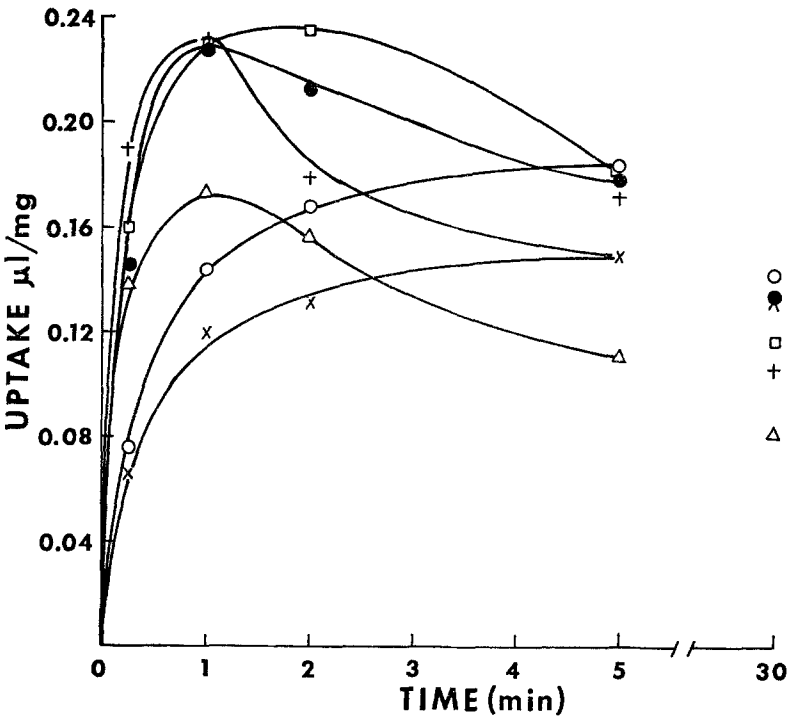

Fig. 7. The pH-dependence of the time course for [1-^{14}C]AIB transport. Na^+ is present outside and K^+ inside the vesicles. The medium pH was 5.5 (×), 6.0 (○), 7.0 (●), 7.5 (□), 8.0 (+), and 9.0(△). *See text* for experimental detail. The specific activity and concentration of AIB were the same as in Fig. 1

increasing pH, a maximum being reached at pH 8. (2) The ability to retain intravesicular AIB is also a function of pH, the maximum level of accumulation still maintained at 30 min being at pH 6.

Both the increased rates of uptake and loss of AIB at increasing pH could be due to an increase in the rate of Na^+ uptake and K^+ efflux at higher pH's. Thus, an increase in rate of Na^+ uptake would (1) decrease the time over which ion gradients were present and (2) increase AIB influx. Intravesicular AIB would reach a higher level sooner, and then start to decay. Alternatively, the effect of pH could be associated with an effect on the carrier mechanism for AIB. To distinguish between these possibilities, the effects of pH on Na^+ influx were examined. No experimentally significant difference in Na^+ influx was found between pH 6 and 8.0. These results suggest an action of pH on the mechanism for AIB transport. The possibility must also be considered that pH gradients may influence AIB transport directly. This possibility is currently under investigation.

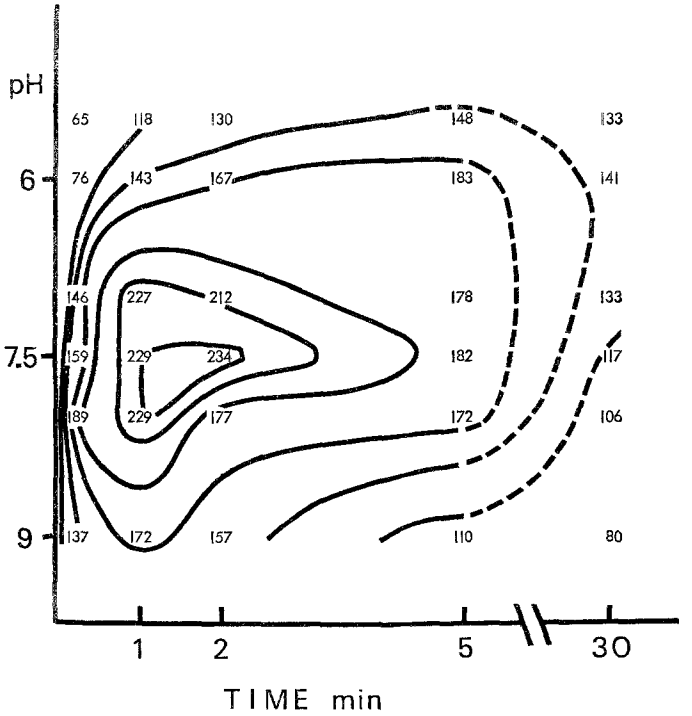

Fig. 8. Contour map showing the pH-dependence of the time course for [1-^{14}C]AIB uptake. Na^+ is present outside the vesicle and K^+ inside throughout. The ordinate shows the pH value of the external medium. The numbers in the graph show the levels of uptake expressed as nl/mg protein. The lines are the positions of equal uptake. The concentration and specific activity of AIB were as shown in Fig. 1

Effect of Ionophores on Gradient-Stimulated AIB Uptake

Gramicidin is an ionophore which is believed to form pores in membranes greatly accelerating the movement of alkali metal cations including Na^+ or K^+ (Miller & Rudin, 1967). Hence, when present, it will accelerate the dissipation of Na^+- and K^+-gradients, bringing these ions to an equilibrium position more rapidly. When gramicidin was added during gradient-stimulated AIB uptake, it reduced the extent of stimulation of AIB uptake and decreased the time required to attain the final steady-state position (Fig. 9). The latter phenomena correlated with an accelerated rate of $^{86}Rb^+$ exit. However, gramicidin did not completely abolish AIB accumulation against a gradient. The results are consistent with the conclusion that the presence of a Na^+ electrochemical gradient is required for the stimulation of AIB uptake, but do not eliminate the possibility that other energy sources such as the ATP found associated with the vesicles (Colombini &

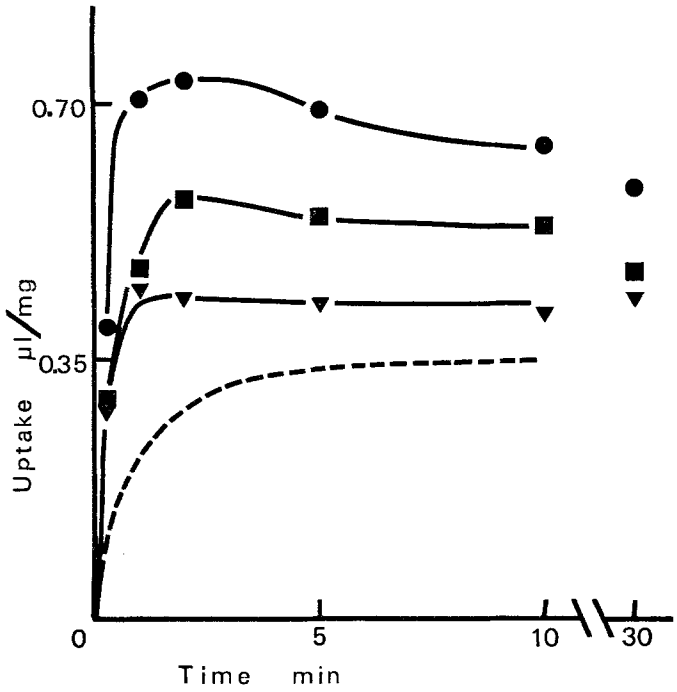

Fig. 9. Ionophores and transport of [1-^{14}C]AIB. Na^+ outside and K^+ inside the vesicles throughout. (■) control, (▼) 2 μg/ml gramicidin D, and (●) 5 μg/ml valinomycin. The level of AIB uptake when Na^+ is present on both sides is shown by the dotted line. The concentration and specific activity of AIB were as shown in Fig. 1. The final ethanol concentration present in (●, ▼) was 0.1 % (v/v)

Johnstone, 1974) are also available to sustain AIB accumulation. The data in Fig. 9 are in agreement with our previous findings (Colombini & Johnstone, 1974) that gramicidin does not decrease AIB uptake in an all-Na^+ medium where a twofold accumulation of AIB is obtained.

In contrast to gramicidin, the ionophore, valinomycin, which is quite specific for the translocation of K^+, stimulates the accumulation of AIB in response to the cation gradients (Fig. 9). A similar result has been observed with intact cells (Gibb & Eddy, 1972). The increased uptake of AIB in the presence of valinomycin is probably due to the production of a K^+ diffusion potential which in turn increases the electrochemical potential for the Na^+ ion.

Action of Ouabain

Although ouabain has frequently been stated to act specifically on the $Na^+ + K^+$-ATPase, the fact that it also affects other transport systems

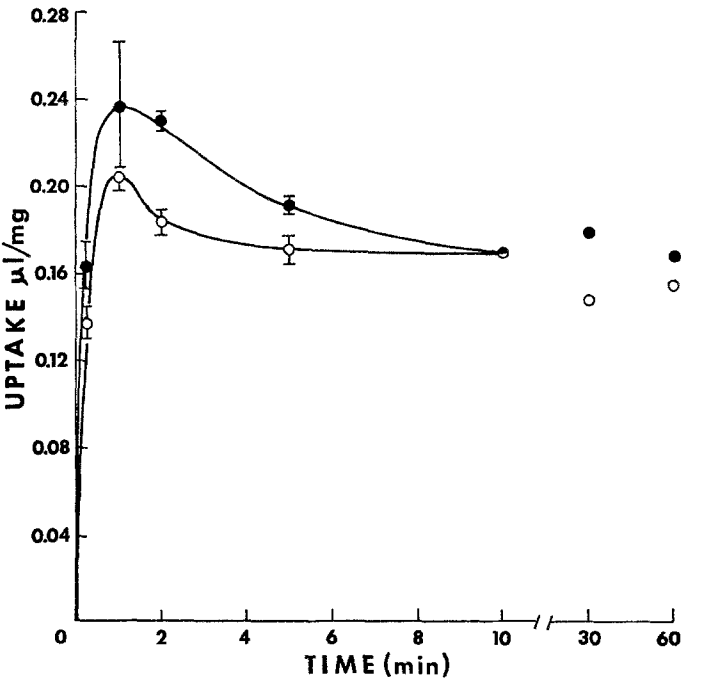

Fig. 10. Effect of 1.0 mM ouabain on [1-^{14}C]AIB transport in the presence of ion gradients. (Na^+ outside and K^+ inside.) The values shown are means of two experiments with (○) and without (●) ouabain. The vertical bars show the spread in values. The specific activity and concentration of AIB were as given in Fig. 1

raises the possibility that it may have a more direct action on the latter transport systems. However, discrimination between a direct and an indirect action of ouabain on organic solute transport is not simple since most systems studied also transport Na^+, and Na^+ movement is coupled to the transport of organic solutes. Using plasma membrane vesicles, we have not been able to show any action of ouabain on cation movements nor on their steady-state distributions. However, addition of ouabain causes an inhibition of AIB uptake (Fig. 10). This action of ouabain is obtained despite the fact that preincubation with ouabain is carried out in the presence of high K^+. It is known that K^+ counteracts the effect of ouabain (Skou, 1957). Therefore these data provide the first direct demonstration that ouabain may be a more general inhibitor of Na^+-dependent transport systems than has heretofore been assumed.

Discussion

In recent years the coupling of Na^+ flux with that of an organic solute has been the focal point of much research since Na^+ dependence is fre-

quently associated with energy-dependent accumulation of organic solutes in animal cells. We have obtained vesicles prepared from plasma membranes of Ehrlich ascites cells which do not appear to be capable of glycolysis or respiration but show Na^+-dependent transport of AIB against its chemical gradient. This conclusion is supported by the fact that (1) accumulation is greater in Na^+ than in choline, K^+, or sucrose media (Colombini & Johnstone, 1974). (2) AIB accumulation in the vesicle is greater than that for Na^+, Cl^-, Rb^+, or 3-O-methylglucose. (3) The accumulation can be increased by the imposition of a Na^+-gradient. The uptake of 3-O-methylglucose and Na^+ is little affected by the imposition of ion gradients.

The nature of the counterion (intravesicular ion) does not play a critical role in stimulating the rate of AIB accumulation. Even replacement of an alkali metal ion with ethanolamine results in an enhanced AIB uptake. These data suggest that AIB uptake in these vesicles is coupled only to the Na^+-gradient and leads us to conclude that only the energy from the Na^+ electrochemical potential difference is used for coupled transport.

Since quantitative differences in the rate of AIB transport are observed with different intravesicular cations, diffusion potentials whose magnitude depends on the mobility of the counterion used, may be induced. The order $Cs^+ > K^+ > Li^+$ would be expected from the known mobilities of these ions in free solution and we find that this is the order in which these ions, when used as counterions to Na^+, stimulate AIB uptake (Fig. 4). In this manner the Na^+ electrochemical potential is partly determined by the intravesicular ion and AIB influx may be indirectly influenced by the nature of the intravesicular ion.

Although it is by no means certain that the only mechanism by which the counterion influences AIB accumulation is through an increase in the electrochemical potential difference for Na^+, all the data are consistent with this possibility including the actions of ethanolamine and valinomycin.

The behavior of the vesicles in response to the ion gradients is reminiscent of that seen in intact cells depleted of ATP where a transient accumulation may be obtained during cation fluxes. However, unlike ATP-depleted intact tumor cells (Eddy, 1968; Potashner & Johnstone, 1971) or intestinal mucosa (Goldner, Hajjar & Curran, 1972), we have not been able to obtain reversed flow of organic solute (AIB) against its concentration gradient by reversing the direction of the cation gradients (results not shown). Transient loss of vesicular AIB is obtained when vesicles, which have been brought to steady state in an all Na^+ medium, are switched to a K^+ medium while maintaining the same extravesicular concentration of AIB. Since the vesicular AIB concentration in an all Na^+ medium is about twice the

extravesicular concentration, this represents a loss along the concentration gradient. One cannot help but speculate whether the ATP found associated (Colombini & Johnstone, 1974) with these membranes is instrumental in preventing loss of AIB against a concentration difference. Further experiments are required to test this possibility and to determine whether the gramicidin-insensitive accumulation is also dependent on this residual ATP level. Preliminary experiments with intact cells have shown that even in the presence of gramicidin a small accumulation of AIB and glycine (1.5 × the medium concentration) is obtained so long as glucose is present to increase the ATP level. No accumulation is observed in the absence of glucose where the ATP levels are less than 0.05 mM (R. M. Johnstone, *unpublished observations*).

The response of the ion gradient-stimulated AIB uptake to pH is consistent with an action of H^+ ions on the carrier mechanism since the rate of dissipation of the ion gradients is unaffected by pH. The fact that both the rate of uptake and the loss of AIB are increased at higher pH values suggests that either the affinity for AIB is increased or that the velocity of translocation is increased in both directions. So long as the driving force of the ions is such that AIB accumulation is possible, the rate of uptake is increased. When the gradient is minimal, however, uptake cannot be sustained and exodus occurs at a greater rate at the higher pH values.

In conclusion, we have obtained a subcellular system which shows many of the transport properties of the intact cell and in which an unequivocal dependence of AIB accumulation on the electrochemical potential difference for Na^+ is obtained.

Appendix

$$[K_i^+]=\frac{K^+ \text{ moles}_{(i)}}{V_i} \rightarrow \therefore V_i=\frac{K^+ \text{ moles}_{(i)}}{[K_i^+]} \tag{6}$$

where V_i = intravesicular volume and (i) refers to intravesicular.

$$[Na_i^+]=\frac{Na^+ \text{ moles}_{(i)}}{V_i}=\frac{Na^+ \text{ moles}_{(i)}[K_i^+]}{K^+ \text{ moles}_{(i)}} \tag{7}$$

but

$$[Na_i^+]+[K_i^+]=0.1 \text{ M} \rightarrow [K_i^+]=0.1-[Na_i^+]. \tag{8}$$

$$\begin{aligned}\therefore [Na_i^+] &= \frac{Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}}(0.1-[Na_i^+]) \\ &= 0.1\frac{Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}}-\frac{Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}}[Na_i^+]\end{aligned} \tag{9}$$

$$\therefore [Na_i^+]\left(1+\frac{Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}}\right)=0.1\frac{Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}} \tag{10}$$

$$\therefore [Na_i^+]=\frac{0.1\ Na^+ \text{ moles}_{(i)}}{K^+ \text{ moles}_{(i)}+Na^+ \text{ moles}_{(i)}} \tag{11}$$

$$\therefore \frac{[Na_o^+]}{[Na_i^+]}=\frac{[Na_o^+]\,(K^+ \text{ moles}_{(i)}+Na^+ \text{ moles}_{(i)})}{0.1\ Na^+ \text{ moles}_{(i)}}. \tag{12}$$

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