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Na^+-Dependent Amino Acid Transport in Plasma Membrane Vesicles from Ehrlich Ascites Cells

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Summary. Purified mammalian plasma membrane vesicles are shown for the first time to transport amino acids. The transport system shows time and temperature dependence. The transport of the nonmetabolizable amino acid, 2-aminoisobutyric acid (AIB) was shown to require sodium ions, potassium and choline ions being unable to substitute. The Na^+-dependent AIB transport was saturable with an apparent Michaelis constant of 3.7 mM. Several observations indicate that AIB is actively accumulated despite an apparent lack of sensitivity to agents which abolish cation gradients or reduce ATP production such as gramicidin A, cyanide and dinitrophenol. Several observations, particularly the sensitivity to increased osmotic strength indicate that the phenomenon under investigation is transport rather than binding of AIB. Evidence that the system is also capable of catalyzing exhange diffusion is demonstrated by the fact that methionine and ethionine in the medium stimulate the efflux of previously accumulated methionine whereas no such effect is seen with AIB.

The analysis of systems which transport organic molecules across biological membranes has been hampered by the necessity to study these systems in the intact tissue or intact cell. Recently, studies on isolated membranes have been undertaken, the greatest progress having been made with prokariotic membranes (Kaback, 1972). Carter and Martin (1969) and Illiano and Cuatrecasas (1971) reported that vesicles derived from plasma membranes of rodent adipocytes were capable of facilitated transport of glucose. Transport of glucose in vesicles and ghosts prepared from human erythrocytes has also been studied (Jung, Carlson & Whaley, 1971; Carter, Avruch & Martin, 1973; Taverna & Langdon, 1973). While some reports (Carter *et al.*, 1973) indicated that treatment with proteolytic enzymes was required to enhance glucose uptake, Jung, Carlson and Balzer (1973) showed that extensive treatment with pronase resulting in removal of 50% of the membrane protein did not result in loss of glucose transport activity.

A Na^+-dependent glucose transport was reported (Busse, Elsas & Rosenberg, 1972) using renal tubule membranes. However, these membranes also metabolized glucose, and Chesney, Sacktor and Rowen (1973) reported that if the tubular membranes are further purified so that they do not metabolize glucose, the Na^+ dependence is lost. A Na^+-dependent transport system for glucose derived from the brush border of intestinal membranes has also been described (Hopfer, Nelson, Perrotto & Isselbacher, 1973). In contrast to these studies, Kahlenberg, Urman and Dolansky (1971) have obtained evidence that in their preparations from human erythrocytes, glucose is bound to membrane proteins, and not transported.

Two studies have appeared on amino acid uptake by isolated animal membranes. The first report concerns proline binding to renal tubule membranes (Hillman & Rosenberg, 1970) using the membrane preparation described for glucose transport (*see* Busse *et al.*, 1972). The second report (Lee, Beygu-Farber & Vidaver, 1973) concerns glycine transport by membrane vesicles prepared from whole pigeon red cells by sonication in which a Na^+-dependent transport of glycine was observed. Competition by alanine and sensitivity to temperature were shown. However, the latter preparation probably contained mitochondria, endoplasmic reticulum and even nuclear membranes, since the data presented showed no decrease in the DNA/protein ratios in the vesicles as compared to the total lysates. Unlike white vesicle and ghost preparations of mammalian red blood cells (Dodge, Mitchell & Hanahan, 1963; Steck, Weinstein, Straus & Wallach, 1970; Perrone & Blostein, 1973) the pigeon vesicles probably still contained hemoglobin as evidenced by the pink color.

We have developed an isolation procedure to produce large quantities of highly purified plasma membranes from Ehrlich ascites tumor cells (Colombini & Johnstone, 1973). This preparation appears relatively free from contamination with other cellular components such as DNA and mitochondria as shown by chemical and electron-microscope examination. Ehrlich ascites cells were chosen because (1) their transport properties have been well studied (Oxender & Christensen, 1963; Johnstone & Scholefield, 1965; Christensen, 1969), (2) these cells have been one of the test systems studied in the current controversy on the energy source of transport processes (Jacquez & Schafer, 1969; Schafer & Heinz, 1971; Gibb & Eddy, 1972; Johnstone, 1972; for Symposium *see* Na-Linked Transport of Organic Solutes (E. Heinz, editor) Springer-Verlag, 1972), and (3) they have both Na^+-independent amino acid exchange and Na^+-dependent transport systems (Inui & Christensen, 1966; Schafer & Jacquez, 1967; Potashner & Johnstone, 1970).

Our results show that the plasma membranes possess transport activity for various amino acids and show both Na^+-dependent and Na^+-independent activity. The system is sensitive to various inhibitors and responds to temperature changes. The uptake observed appears to be a true transport system and not a binding process since it responds to alterations in osmolarity.

Materials and Methods

Isolation of Plasma Membranes

Plasma membranes were purified from Ehrlich ascites cells as previously described (Colombini & Johnstone, 1973). A large scale procedure was used yielding 100 to 200 mg of membrane protein from a single preparation. Cells from 25 tumor-bearing mice were used. Lysing was done with the Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland) with a potentiometer setting of approximately 3.1. This produces somewhat smaller membrane fragments than the syringe method previously described but large quantities of cells can be handled rapidly. Homogenization was performed for 2 to 5 min, lysis being monitored as previously described. The homogenate (500 ml) was layered over four sucrose gradients (800 ml each) and centrifuged in a Sorvall RC-3 fitted with an HG-4L rotor at $3{,}500 \times g$ for 35 min. The lower 2/5 of the gradient contained the plasma membranes, which were treated as described previously except that all fractions were subjected to differential centrifugation to remove nuclear contamination. Centrifugation for 1 min at $3{,}000 \times g$ (tabletop Sorvall fitted with an SS-34 head), pelleted down the remaining nuclei along with some membranes. The bulk of the membranes were left in the supernatant. The final centrifugation was done at $4{,}000 \times g$ instead of $3{,}000 \times g$ as described previously (Colombini & Johnstone, 1973). Membranes were stored at -20 °C in 15% DMSO.

Assay of Transport Activity

A known volume, about 15 ml, of membrane suspension containing 30 to 40 mg of membrane protein was thawed and centrifuged, 1 ml of 1 M NaCl being added to aid membrane sedimentation, probably by reducing the surface charge (Colombini, *unpublished observations*). Centrifugation was carried out at $10{,}000 \times g$ for 3.5 min on a tabletop Sorvall centrifuge, fitted with an SS-34 head. The supernatant was discarded and the pellet resuspended in 15 ml of 100 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 5 mM Tris Cl, pH 7.5 (20 °C). The suspension was centrifuged as above and resuspended in 3.0 ml of the same solution henceforth known as the standard medium. Resuspension was aided by using a glass stirring rod and by swirling on a Vortex mixer. Care was taken to avoid foaming. Unless otherwise indicated, the suspension was then incubated at 37 °C for 30 min before assaying for transport activity.

Transport measurements were usually done at room temperature (20 °C). Incubation was carried out in a small polyethylene tube. Transport was initiated by adding 5 μliters of ^{14}C substrate per 0.1 ml of membrane suspension. At the specified times the reaction was terminated by removing 0.1 ml of this mixture and injecting it into 1.9 ml of ice-cold standard medium using a biopette (Schwartz-Mann Inc.). The suspension was swirled and immediately filtered under vacuum through a glass fiber filter grade 934AH (Reeve Angel). The lapse of time between initiation of sampling and termination of the first filtration was 5 to 8 sec. The filter was immediately washed with 10 ml of ice-cold

standard solution and then placed into a counting vial containing 10 ml of a modified Bray's solution (Bray, 1960). Zero time samples were done by adding ^{14}C-substrate to the 1.9 ml of cold diluent *followed* by addition of 0.1 ml of membrane suspension and filtering.

Efflux studies were done by first preincubating the membrane preparation with ^{14}C-solute and then diluting 0.1 ml of the sample with 1.9 ml of the standard incubation medium, devoid of the solute in question. The diluted sample was incubated for different periods of time at the temperatures given in the text, then filtered and washed as described in experiments to measure uptake.

Transport activity can be expressed either as moles of substrate taken up per milligram membrane protein per unit time or as microliter equivalents of medium taken up per milligram protein per unit time. In the present context, the latter expression is not to suggest that there has been a net transfer of fluid, but rather represents the microliters of medium cleared of substrate and is a convenient way of normalizing the data from different experiments using a variety of ^{14}C-solute concentrations of different specific activities. The actual moles of ^{14}C-substrate taken up can be obtained by multiplying the microliter equivalents of medium by the concentration of ^{14}C-substrate used in any particular experiment. The expression we used was

$$\frac{\text{cpm per mg protein on the filter}}{\text{cpm per } \mu\text{liter medium}} = \mu\text{liter/mg.}$$

An additional advantage in this method of presentation of the data is that any alteration in retention of radioactivity on the filter which is exactly proportional to a change in the medium radioactivity is not reflected as a change in uptake, thus simplifying comparisons between different experiments using different solute concentrations.

Although different experiments with the same membrane preparation yield very similar levels of uptake when used on different days, different preparations do differ in the extent of substrate trapping by as much as twofold per milligram of protein. Therefore, except where otherwise indicated, all results have been expressed as a percent of the volume taken up at 5 min at 20 °C in the standard medium, since under the latter conditions this system is near the steady state. Protein assay was done by the method of Lowry, Rosebrough, Farr and Randall (1951).

All radioactive chemicals were ^{14}C amino acids purchased from New England Nuclear Corporation. 1-^{14}C-glycine, 1-^{14}C-AIB, methyl-^{14}C-methionine, U-^{14}C-lysine were used. Other reagents were obtained from Sigma Chemical Co., St. Louis, or Fisher Scientific Co.

Results

Amino Acid Transport

Time-dependent amino acid transport was achieved with several but not all amino acids tested (Fig. 1). 2-Aminoisobutyric acid (AIB), glycine and methionine show time dependence while lysine does not. Lysine is trapped on the membranes to a greater extent than are the other amino acids; however this is probably due to the high negative surface charge of these membranes which bind the lysine electrostatically, as evidenced in the proton release accompanying cation binding (*unpublished observations*).

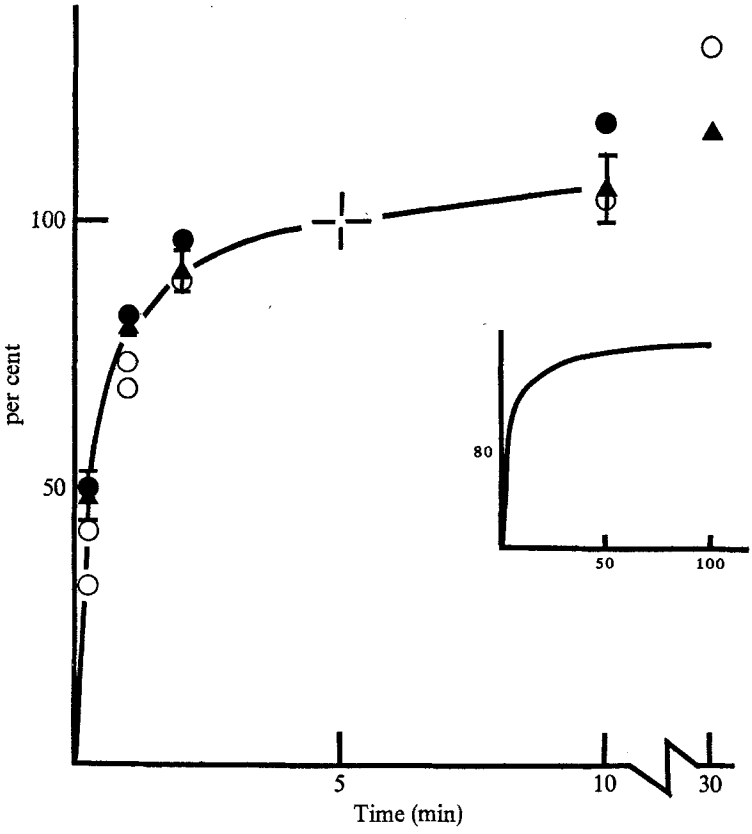

Fig. 1. Amino acid transport as a function of time. Transport is expressed as a percentage of the 5-min value (corrected for zero time): methionine (○), glycine (●) and α-amino-isobutyric acid (AIB) (▲, the vertical bars represent the standard deviation for the AIB uptake). The 5-min uptake for AIB ranged from 0.24 to 0.46 μliter/mg protein (nine experiments), those for methionine were 0.33 and 0.40 μliter/mg (two experiments) and that for glycine was 0.29 μliter/mg (one experiment). Lysine uptake, not shown, was not time dependent but attained a level of 1.0 μliter/mg at zero time. The concentrations of AIB, methionine, glycine and lysine were 0.41, 0.50, 6.0 and 0.10 mM, respectively. The specific activities were 22,000, 13,400, 210 and 15,000 cpm/nmole, respectively. The inset shows a more complete time course for methionine which reached steady state only after 50 min. This final level was 50% higher than the 5-min level

AIB Transport

Most of the subsequent studies were done with AIB as the test substrate, a nonmetabolizable amino acid that is well transported by the intact cell (Inui & Christensen, 1966). The results in Table 1 show that the uptake of AIB by these membranes is almost completely eliminated by boiling or TCA addition. Acidification with hydrochloric acid produces a marked reduction in uptake but some time-dependent uptake remains. The reason for the

Table 1. Effect of inhibitors on AIB transport

Inhibitor	% Decrease in uptake at $t_{(min)}$				
	0.25	1.25	2.0	5.0	10.0
Gramicidin D (12.5 μM)	18	0	−2	−3	–
Phloretin (250 μM)	31	–	–	2	–
$HgCl_2$ (2 mM)	51	–	–	35	–
KCN (1.25 mM)	20	11	17	12	13
DNP (300 μM)	14	−3	12	−1	−2
Na iodoacetate (1 mM)	44	–	–	–	26
HCl (100 mM)	50	–	–	79	–
TCA (5%)	97	–	–	91	–
Boiling (5 min)	84	–	–	–	91

Time courses were done as described in Materials and Methods except as indicated below. Membranes were preincubated for 2 hr at 37 °C with dinitrophenol (DNP) or KCN in the standard medium before transport was measured. The other inhibitors were added a few minutes before transport measurements were initiated. In the boling experiments, the membranes were allowed to cool down to room temperature before making transport measurements. Corrections have been applied for zero time values. Representative data are given. Each inhibitor is compared to a control without inhibitor done in parallel with the same preparation of membranes. Little difference in control uptake was observed if the membranes were incubated for 5 min or 2 hr at 20 or 37 °C prior to initiating transport.

remaining activity is unknown, although it is possible that not all the protein was denatured at pH 2.0.

To test the response of this system to metabolic inhibitors we examined the effects of CN^-, DNP, iodoacetate, gramicidin D, and $HgCl_2$ (Table 1). Of these compounds only $HgCl_2$ and iodoacetate have a pronounced effect on AIB uptake. Gramicidin D is without effect. Phloretin (Beneš, Kalínská & Kotyk, 1972) which has been suggested as a general inhibitor of transport processes in the erythrocyte, does not appear to affect this system (Table 1).

From the data in Fig. 2, it is apparent that influx exceeds efflux by a factor of at least two at 20 °C. Based on the values for AIB uptake after 15 sec to 1 min of incubation, the influx is reduced approximately fourfold by reducing the temperature from 20 °C to 0 °C (Fig. 2). At 0 °C, AIB uptake continues at a low rate, and after 30 min is still only about 60% of the level achieved at 20 °C in 5 min. At 20 °C, near steady state is attained at 5 min. To check if the uptake at 0 °C would eventually reach the same steady state as at 20 °C we incubated the membranes at 20 °C and allowed AIB to reach its steady state. We then switched the experimental flask to a bath at 0 °C, and continued the incubation. After 30 min at 0 °C there is no significant loss of ^{14}C-AIB from the membranes if ^{14}C-AIB at

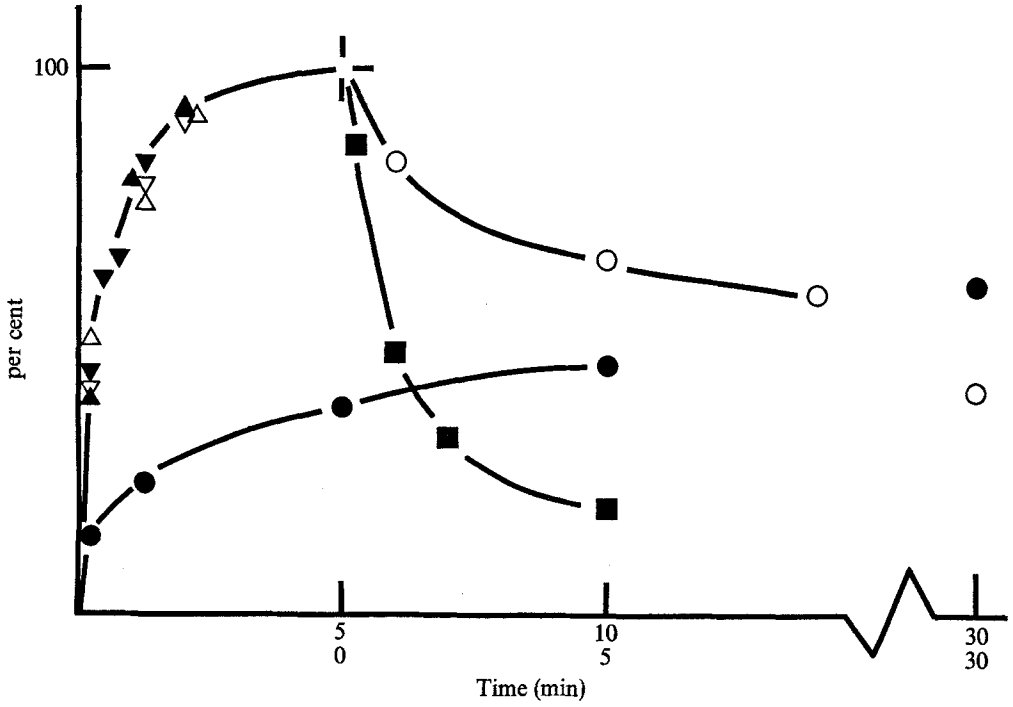

Fig. 2. Uptake (▲, ▼, △, ▽, •) and exodus (■, ○) of AIB at 20 °C (▲, ▼, △, ▽, ■) and 0 °C (○ •) using 5 mM AIB. On the abscissa, the upper scale is for uptake and the lower one for exodus. The transport is expressed as a percentage of the 5-min uptake at 20 °C which ranged from 0.42 to 0.25 µliter/mg (corrected for zero time) in four separate experiments. The specific activities used were 200 and 1200 (▲) cpm/nmole. Exodus was measured in the absence of added ^{14}C-AIB to the medium, and therefore, at initial times, represents a unidirectional flux. Addition of ^{12}C-AIB to the dilution medium during efflux at 20 °C, does not alter the rate of loss of ^{14}C-AIB from the membranes (results not shown)

the original concentration is present in the medium. If, however, exodus is measured into an AIB-free solution, there is a 50% decrease in membrane-associated AIB in 30 min at 0 °C (Fig. 2). From this result we conclude that the steady state is probably the same at 0 °C or 20 °C since, if the steady state at 0 °C were lower than 20 °C, we would expect a loss of AIB from the membrane preparation upon incubation at 0 °C.

The ionic requirements for the transport of AIB were also examined. Removal of Ca^{++} and Mg^{++} from the incubation media has little or no effect. The replacement of Na^+ by choline, however, markedly reduces the transport activity (Tables 2 and 3).

To ascertain whether the required ion in the medium is specifically Na^+ or any alkali metal ion, K^+ was substituted for Na^+ (Table 2). Total replacement of Na^+ by K^+ dramatically changes the course of uptake. At

Table 2. The monovalent ion requirement for AIB uptake

Conditions	AIB Uptake at $t_{(min)}$		
	0.25	10.00	Δ Uptake[a]
	(μliter/mg protein)		
100 mM NaCl	0.092	0.340	0.248
75 mM NaCl, 25 mM KCl	0.121	0.361	0.240
100 mM KCl	0.188	0.224	0.036
100 mM Choline chloride	0.045	0.138	0.093
200 mM Sucrose	0.027	0.085	0.059

The membranes were preincubated for 30 min at 37 °C, as usual, then centrifuged in small polyethylene centrifuge tubes for 3.5 min in an Eppendorf microfuge at 10,000 × *g* and the contents of each tube were resuspended in a medium containing 5 mM Tris Cl (pH 7.5) and the substance shown under "conditions" in the Table. The centrifugation and resuspension step was repeated a second time to assure complete removal of the standard medium. The results have been corrected for zero time values. To convert the values to nmoles/mg protein, the figures in columns 2 and 3 are multiplied by 0.41, the AIB concentration used. The specific activity of AIB was 22,000 cpm/nmole.
[a] Δ uptake = 10 min uptake − 0.25 min uptake.

Table 3. The effect of [AIB] on the steady-state level of AIB uptake

Medium [AIB]	Medium used[a]	Uptake	
(mM)		(μliter/mg)	(nmoles/mg)
0.41	Na^+	0.456	0.187
0.86	Na^+	0.334	0.288
2.21	Na^+	0.328	0.730
4.01	Na^+	0.254	1.02
0.41	Choline	0.193	0.079
9.41	Choline	0.191	1.80

The uptake shown is the steady-state level as measured at 10 min. The membranes were transferred to the choline medium as described in the legend to Table 2.
[a] Na^+ indicates standard medium. Choline indicates 100 mM choline Cl, 5 mM Tris Cl, pH 7.5.

15 sec the uptake in K^+ is double that with Na^+, but with K^+ there is little further change in uptake with time of incubation up to 10 min. The high uptake at 15 sec may be due to K^+-mediated swelling of the intravesicular space. This is not an unreasonable suggestion since whole Ehrlich ascites cells swell in K^+ (Maizels, Remington & Truscoe, 1958; Schafer & Heinz, 1971). When 100 mM NaCl is replaced by 75 mM NaCl and 25 mM KCl, AIB uptake at 10 min is similar to the control values in 100 mM Na^+, but at 15 sec, the AIB associated with the pellet is greater in 25 mM K^+ than

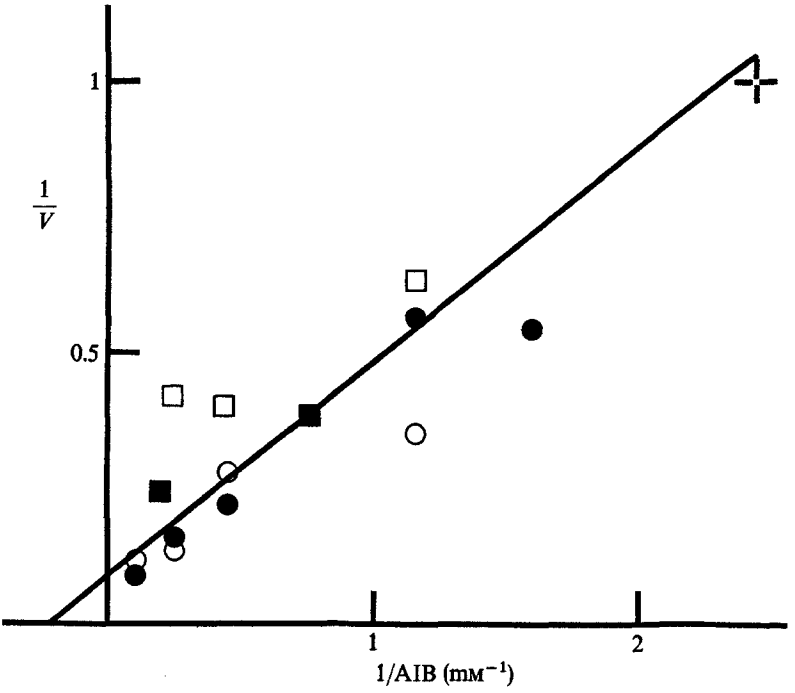

Fig. 3. Lineweaver-Burk plot for Na^+-dependent AIB transport. The $1/V$ values are expressed as a fraction of the $1/V$ value at 0.41 mM AIB (shown by cross) which ranged from 8 to 23 min/nmole. The line drawn was determined by the least-squares fit. The values represent only the Na^+-dependent uptake, values for uptake having been corrected for uptake in choline medium

in zero K^+, suggesting a similar tendency to that seen in 100 mM K^+ medium. It may be noted that despite the initial large uptake in K^+ there is less increase with time in AIB associated with the vesicles in K^+ than in choline medium.

Increasing the medium AIB concentration in a Na^+ medium does not result in a proportional increase in AIB associated with the vesicles (Table 3). That this phenomenon is not due to a decreased intravesicular volume is shown by the fact that in a choline medium the AIB associated with the membranes is directly proportional to the AIB concentration, suggesting a maximal Na^+-dependent accumulating capacity.

The saturability of the transport system was examined by measuring the initial rate of AIB uptake as a function of AIB concentration. Fig. 3 shows a Lineweaver-Burk plot of the Na^+-requiring component of the transport system which has been corrected for the uptake in a choline medium. Fifteen-second incubation values were used to obtain this double reciprocal plot in order to obtain values approximating the initial rates. Although the values

show scatter, the time of incubation being of short duration (15 sec), the data from four different experiments cluster around a single straight line. If individual experiments are examined the deviation from linearity is less. By using the method of the least-squares fit, the apparent K_m is calculated to be 3.7 mM. It is evident that the system is saturable. It may be noted that Inui and Christensen (1966) reported a K_m value of 2.0 mM for AIB transport in Ehrlich cells.

One of the problems associated with experimental work with vesicles is the difficulty of differentiating between transport into an intravesicular space and binding to the membrane proteins. Since the vesicles are usually small with a large protein-to-volume ratio, sufficient numbers of sites could theoretically be available on the proteins (if a large percentage of membrane protein were amino acid binding protein) to bind the experimental solute and account for the "uptake". One approach to differentiate between binding and accumulation is to examine the response to changes in osmolarity. Under conditions of constant ionic strength, solutes which do not penetrate the vesicular space would be expected to reduce the intravesicular volume, hence reducing uptake associated with the "filling of a space" without affecting binding to available protein sites. In our experiments osmotic pressure was increased by sequential addition of sucrose. As sucrose concentration increased, the amount of AIB associated with the vesicles decreased (Fig. 4). Almost all the AIB associated with the vesicles is released if sufficient sucrose is added (240 mM sucrose). In contrast, urea, which is expected to be much more permeable, has much less effect. Urea would be expected to reduce nonspecific binding of AIB by interfering with H-bonding and substrate-ligand binding more effectively than sucrose. The mechanism by which urea, at concentrations in excess of 50 mM, reduces AIB uptake could be due to a direct inhibition of the transport system, or the fact that some shrinkage has occurred because of incomplete penetration. To test these possibilities we compared the action of sucrose and urea on AIB uptake in a choline medium in lieu of the NaCl medium. The data in Fig. 4 (inset) show clearly that the inhibition by urea, in contrast to that by sucrose, is not seen in a choline medium. These data support the conclusion that AIB is being transported into an intravesicular space. The reduced inhibitory effect of sucrose in a choline medium compared to a NaCl medium is due to the fact that the 100% value for uptake in choline is only 60% of that seen in NaCl. Based on a calculation of micromoles of AIB per milligram protein, nearly equal amounts of AIB are associated with the membrane in NaCl or choline upon addition of 200 mM sucrose. It should be noted that if the NaCl is removed and 200 mM sucrose is used to maintain osmolarity,

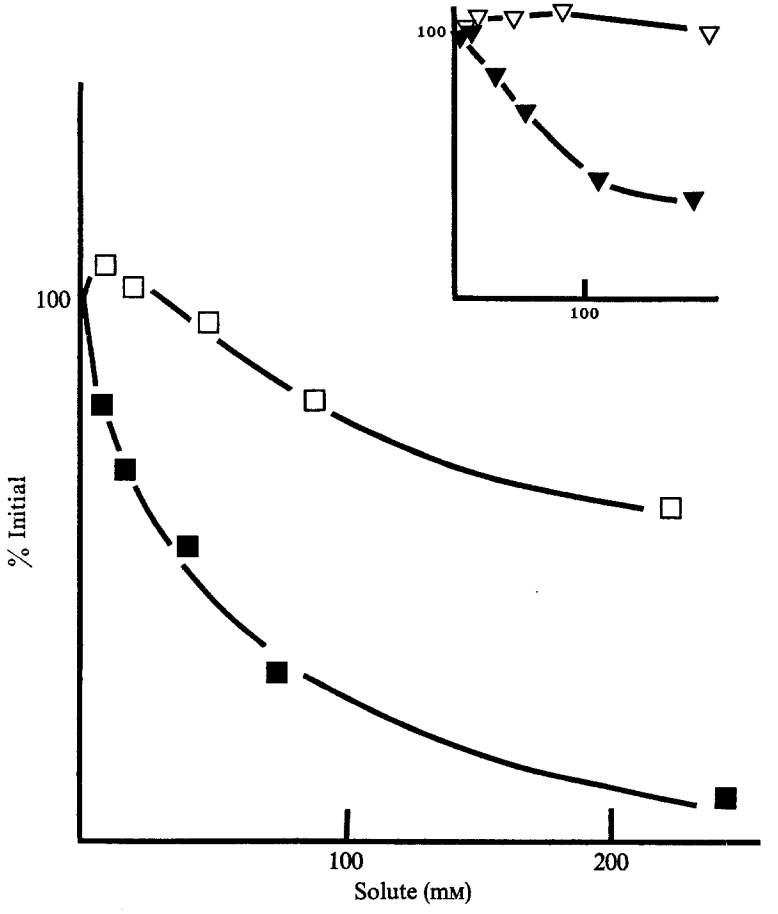

Fig. 4. Effect of osmotic strength on AIB uptake. After allowing the AIB to reach steady state (10 min), sucrose (■, ▲) or urea (□, △) was added and a sample was taken after 5 min. Then a second aliquot of the same substance was added followed by sampling 5 min later. This was continued until a solute concentration of about 200 mM was reached. The inset shows the behavior of the system when the standard medium is replaced by 100 mM choline Cl, 5 mM Tris Cl, pH 7.5. The initial uptakes ranged from 0.23 to 0.42 μliter/mg protein. The AIB concentration used was 0.41 mM and the specific activity was 22,000 cpm/nmole

the uptake observed is greater than that seen when sucrose is added in addition to 100 mM NaCl. Therefore, the results are not due to an unspecific inhibition by sucrose.

Methionine Transport

From Fig. 1, it is clear that a time-dependent uptake of methionine occurs in these vesicles. It is known (Johnstone & Scholefield, 1961) that methionine is capable of exchanging with other amino acids in the intact

Table 4. Amino acid-stimulated methionine efflux

Exp. No.	Temperature (°C)	Additions	% Lost at $t_{(min)}$			
			0.25	0.50	2.0	30
Exp. 1	20	Control	33	41	44	74
	20	+10 mM methionine	36	46	53	77
	0	Control	4	—	15	56
	0	+10 mM methionine	16	—	28	63
Exp. 2	0	Control	−2	8	24	57
	0	+10 mM methionine	10	16	34	55
	0	+10 mM ethionine	9	19	34	58
	0	+10 mM alanine	2	13	27	53
	0	+10 mM glycine	4	15	26	57

The efflux studies were done as described in Materials and Methods. The amino acid concentration under "additions" is the final concentration of the ^{12}C-amino acid added to the dilution medium. The control uptakes varied from 0.48 to 0.65 μliter/mg protein. Since the methionine concentration in the incubation medium was 0.5 mM, 0.24 to 0.32 nmoles/mg protein were taken up by the control preparations at steady state. The specific activity of the ^{14}C-methionine used was 13,400 cpm/nmole.

cell and we therefore attempted to demonstrate this property in the vesicles. The effect of amino acids in the medium on the efflux of methionine from prepacked vesicles was examined (Table 4). Methionine was shown to stimulate efflux both at 20 °C and 0 °C although the effect was more pronounced at 0 °C. The differences at 20 °C albeit small, are consistent. Ethionine was almost as effective as methionine at stimulating the efflux while glycine and alanine were less effective.

Discussion

The present report provides evidence of a transport system for amino acids with properties similar to those of the intact cell. The actions of $HgCl_2$ and iodoacetate suggest a requirement for an SH group for transport activity.

The possibility that binding, and not accumulation, is occurring seems unlikely since increasing the osmotic pressure with sucrose decreases the transport activity whereas addition of urea is much less effective. Since it would be anticipated that binding would be reduced more markedly by urea than by sucrose and more by a charged than an uncharged species, the actions of urea, choline and sucrose on the system are consistent with an accumulation rather than binding.

The effect of temperature on the system is also inconsistent with binding since the latter process would be expected to increase as the temperature is decreased. The fact that methionine or ethionine in the medium stimulate the loss of "vesicular" methionine in a specific manner also supports the conclusion that methionine is transported across the vesicular membrane rather than bound to it. Studies with intact cells have demonstrated that methionine and ethionine exchange more readily with cellular methionine than do glycine and alanine (Johnstone & Scholefield, 1961).

If the solute is dissolved in the intravesicular fluid the question arises whether the AIB (or the other amino acids) exists in the vesicular space at a higher concentration than in the incubation medium. It is difficult to ascertain conclusively whether in a Na^+ medium the amino acid is present at an elevated concentration in the vesicle, although several lines of evidence suggest that it is. First, AIB is a neutral molecule at pH 7.4. Therefore, if the AIB in the vesicle were in equilibrium with the medium, the steady-state level of AIB associated with the vesicle should be proportional, in a linear manner, to the AIB concentration in the medium. That this is not so is shown by the observation in Table 3 that in a Na^+ medium, AIB associated with the vesicle does not increase proportionally to the medium concentration. Second, in a choline medium the above prediction is correct, the AIB in the vesicle at steady state being directly proportional to the medium AIB concentration (Table 3). Since the amount of AIB associated with the vesicle in a Na^+ medium at steady state is about twice that in a choline medium, the concentration of AIB in the vesicles in a Na^+ medium may be twice that in a choline medium if it is assumed that in the choline medium the intravesicular AIB concentration is equivalent to the medium AIB concentration. The other possibilities are that in a choline medium the intravesicular AIB concentration is less than that of the medium, despite the experimental observation that the system is at steady state (results not shown) or there is a decrease in vesicular volume in choline chloride. Third, if the transport system were an equilibrating one, the flux in one direction should be equal to the flux in the opposite direction at the same [AIB]. The data however show that influx from a 5-mM AIB solution is at least twice the efflux, using vesicles which were preincubated with 5 mM AIB (Fig. 2).

While none of these lines of evidence is conclusive they all suggest that in standard medium, containing 100 mM NaCl, the concentration of AIB in the vesicles may attain levels which are higher than in the medium. If this interpretation is correct, the question of an energy source for the process is raised. We have not as yet measured the cation distributions in this system, and therefore we cannot eliminate the possibility that an ion

gradient exists and provides energy. The lack of effect of gramicidin D, however, argues against this possibility since it would be expected to dissipate existing monovalent cation gradients (Henderson, McGivan & Chappell, 1969). The small effects of DNP and CN^- argue against a dependence on respiratory activity and oxidative phosphorylation. However, when this membrane preparation was assayed for ATP using the firefly assay (Stanley & Williams, 1969) 5 pmoles of ATP were found per mg protein. The metabolic source of ATP and the reason for its lack of destruction by the high membrane ATPase (Colombini & Johnstone, 1973) are at present obscure.

In conclusion, the data are consistent with the interpretation that we have obtained a relatively pure preparation of plasma membranes which is capable of accumulating amino acids by a process that is Na^+-dependent and probably energy-dependent and which is also capable of catalyzing exchange diffusion of amino acids.

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