Biochimica et Biophysica Acta, 599 (1980) 175—190

BBA 78782

ENERGIZATION OF AMINO ACID TRANSPORT IN ENERGY-DEPLETED EHRLICH CELLS AND PLASMA MEMBRANE VESICLES

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(Received October 1st, 1979)

Key words: Amino acid transport; Energy depletion; Membrane vesicle; ATP level; (Ehrlich ascites)

Summary

We redirect attention to contributions to the energization of the active transport of amino acids in the Ehrlich cell, beyond the known energization by down-gradient comigration of Na^+, beyond possible direct energization by coupling to ATP breakdown, and beyond known energization by exchange with prior accumulations of amino acids. We re-emphasize the uphill operation of System L, and by prior depletion of cellular amino acids show that this system must receive energy beyond that made available by their coupled exodus. After this depletion the Na^+-independent accumulation of the norbornane amino acid, 2-aminobicycloheptane-2-carboxylic acid becomes strongly subject to stimulation by incubation with glucose. Energy transfer between Systems A and L through the mutual substrate action of ordinary amino acids was minimized although not entirely avoided by the use of amino acid analogs specific to each system.

When 2,4-dinitrophenol was included in the depleting treatment, and pyruvate, phenazine methosulfate, or glucose used for restoration, recovery of uptake of the norbornane amino acid was independent of external Na^+ or K^+ levels. Restoration of the uptake of 2-(methylamino)isobutyric acid was, however, decreased by omission of external K^+. Contrary to an earlier finding, restoration of uptake of each of these amino acids was associated with distinct and usually correlated rises in cellular ATP levels. ATP addition failed to stimu-

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Abbreviations: BCH, 2-aminobicycloheptane-2-carboxylic acid (2-aminonorbornane-2-carboxylic acid) in this paper only, the b(—) isomer; MeAIB, 2-(methylamino)isobutyric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

late exodus of the norbornane amino acid from plasma membrane vesicles, although either NADH or phenazine methosulfate did stimulate exodus. ATP production and use is thus associated with transport energization, although evidence for a direct role failed to appear.

Introduction

The Na^+-dependent transport of neutral amino acids across the plasma membrane of Ehrlich ascites tumor cells can be driven by linked down-gradient flows of Na^+ [1—5]. Because of our part in their discovery our inclination might well be to find these linked flows a sole and adequate source of energization. Nevertheless the occasionally urged and well-supported possibility of significant supplementation from other sources of energy should not be disregarded. Because the comigration of the test amino acid with Na^+ is electrogenic, an important question is the magnitude of the transmembrane potential across the interval where this linked comigration with Na^+ actually occurs. Through the electrogenic character of the ($Na^+ + K^+$)-ATPase pump, the electrochemical potential of Na^+ may be maintained at values higher than are ordinarily estimated [6,7]. Recent estimates with a fluorescent probe still leave, nevertheless, a significant deficit from the gradient calculated to be needed to maintain the observed methionine gradient in an ascites tumor cell somewhat different from the Ehrlich cell. The two gradients varied together in a nearly linear way, however, over wide ranges [8,9], the methionine gradient remaining 1.8-times as large as the estimated electrochemical gradient for Na^+.

Stimulated by the observations of Schafer and his associates [10,11] that concentrative uptake of 2-aminoisobutyric acid persists after alkali-ion gradients have been made unfavorable and ATP levels very low by inhibitors of energy metabolism, we have shown that the concentrative uptake of amino acids both by the Na^+-dependent and the Na^+-independent routes can be restored by adding pyruvate, an ascorbate/phenazine methosulfate combination, or glucose [12,13]. These results occurred before the chemically determined gradients of the alkali ions rose and while the rise in the ATP level was still small. These results emphasized the possibility that concentrative uptake of amino acids by the several systems can be energized by some means in addition to the down-gradient flows of alkali ions and a hypothetical coupled hydrolysis of ATP. A failure so far to find amino acid-dependent ATPase activity consistent with any of the principal transport systems is also pertinent. Experiments by Geck et al. [14] have indicated that ATP hydrolysis probably does not directly energize Na^+-dependent amino acid transport. Reid et al. showed that under a favorable Na^+ gradient an ascites tumor cell depleted of ATP accumulates approximately as much methionine as do respiring cells [15].

We believe in addition that Na^+-independent System L deserves parallel attention when energization is being considered, although summaries of the energetic question sometimes ignore the whole problem of catalytic heterogeneity in amino acid transport. Rarely do natural amino acids have their transport restricted to either System A or L, and the cooperation that can occur between the two systems is often overlooked. We showed some years ago for the Ehrlich

cell extensively depleted of exchangeable amino acids that in the absence of Na^+ the concentrative uptake either of the norbornane amino acid (BCH) in the isomeric form b(±), or of another model amino acid whose uptake we restricted to System L, was far larger than the total concurrent loss of especially exchangeable amino acids from the cell (Ref. 16, Fig. 8). We now reinforce these measurements by showing a 5 min uptake of the b(−) isomer of the same amino acid over ten times the induced concurrent loss from the Ehrlich cell of all free amino acids measured by the amino acid analyzer. These results mean that uphill transport by System L must be energized in a way other than by Na^+ cotransport or the down-gradient exodus of endogenous amino acids. They point to another more fundamental source for the energy inherent in the concentration gradients established for any amino acid even moderately reactive with System L.

System L needs to be taken into account not only for its independent energization but also for its service in transfer and conservation of energy inherent in the interaction between parallel transport systems. This function is most easily demonstrated by providing in the external environment another amino acid (e.g., tryptophan), in addition to one concentrated well by both Systems A and L, say methionine (Ref. 17, Fig. 6). In this situation one can intensify the accumulation of methionine by blocking participation of System L, which has so far been accomplished by supplying BCH at balanced concentrations on two sides of the plasma membrane. Coincidentally, the uptake of tryptophan is decreased. The result showed that net entry of methionine at the steady state occurred by System A, net exodus by System L [17]. The energy available from methionine exodus was conserved in part by its driving of tryptophan entry. Although net exodus of other amino acids by System L from the Ehrlich cell has been shown [18], we have not yet formally demonstrated this circuit, inflow by System A and energy-conserving outflow by System L, as prevailing for methionine, for example, when supplied alone. For that demonstration, we should have an irreversible inhibitor of System L which places no significant energetic load on the membrane.

From the foregoing considerations, it is not plausible to count the exodus of methionine by a reversed operation of System L as a simple leak (although the correction for methionine leakage applied by Philo and Eddy probably had that effect [8,9]) because by the argument of the preceding paragraph that exodus is probably doing work. Conservation of energy in this way may account for the energetic deficiency noted in Philo and Eddy's work, the methionine gradient having been sustained at a value consistently higher than that predicted from the calculated electrochemical gradient of Na^+. This criticism would not apply to related observations with 2-(methylamino)isobutyric acid (MeAIB) in the Ehrlich cell.

The additional alternative is that System L channels energy supplied primarily to it to enhance the accumulation of methionine presented alone, above the gradient produced by System A. For methionine this effect seems unlikely to us at normal Na^+ levels, because then System A would produce methionine gradients that System L could probably not sustain and almost certainly not intensify. At lower external levels of Na^+, however, we suggest that the situation might well be reversed, with the Na^+-independent system now producing a

gradient greater than System A can sustain. The Na^+ level at which the shift in A and L roles occurs would be expected to vary among the amino acids.

To minimize cooperative effects between Systems A and L of the sort that may have added complications to the experiment of Philo and Eddy, in the present tests we have used two model amino acids, MeAIB and BCH, which in our cell line give consistent evidence of transport limited to System A and System L, respectively. Inherent, however, in the phenomenon of competitive stimulation [17] is the expectation that BCH uptake will as is observed show more and more Na^+ stimulation as the interval of uptake increases, given that any dual-affinity amino acid is thus given time to cycle between the interior and exterior of the cell. In the experiments described here with the Ehrlich cell and plasma membrane vesicles derived from it, we present further evidence that down-gradient flows of alkali ions are not a sufficient driving force for the restoration of amino acid transport after an energetic depletion that has largely removed ATP. We show that the production of new ATP is linked to the restoration, even though the energy invested in that substance does not seem, as already urged by others, to be used directly in energizing neutral amino acid transport.

Experimental procedure

Materials. The amino acids, from standard commercial sources or synthesized in this laboratory, were extensively studied preparations. [1-^{14}C]MeAIB was obtained from New England Nuclear Corp., Boston, MA, U.S.A. [^{14}C]BCH, as the b(−) isomer, was prepared in this laboratory [19]. Metabolic inhibitors were used from commercial sources except for fluoromalate kindly given to us by Dr. Ernest Kun, Cardiovascular Research Institute, University of California Medical Center, San Francisco.

Measurement of amino acid uptake by depleted Ehrlich cells. The cells were collected and handled as described previously [12,20—22]. The Ehrlich ascites tumor cells were collected from male Swiss white mice 8—10 days after inoculation with the tumor cell. To lower their content of endogenous amino acids, either of two procedures was used:

(1) The washed cells were incubated in three successive periods of 10 min each at 37°C in a modified Krebs-Ringer bicarbonate medium (pH 7.4) in which all but 6 mM of the Na^+ had been replaced by choline. For convenience these solutions and the three following washes with Krebs-Ringer bicarbonate medium with a normal Na^+ content were buffered to pH 7.4 with 15 mM bicarbonate and 12 mM phosphate, rather than with bicarbonate alone.

(2) The washed cells were incubated 15 min in a 6-fold volume of hypo-osmotic Krebs-Ringer/Hepes medium, pH 7.35 (30 mM, NaCl, 35 mM KCl, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 12.5 mM Hepes), and then 15 min in a Krebs-Ringer/Hepes medium of normal tonicity. These cells were then depleted of ATP by a 30 min incubation in Krebs-Ringer bicarbonate buffer containing 0.2 mM dinitrophenol.

Restorative treatment. After the depleting treatments, cell damage was checked by trypan blue staining. The percentage of cells now staining with trypan blue was shown not excessive, e.g. 8.8 ± 1.3% for the second procedure including the dinitrophenol treatment. Subsequently, 0.3-ml aliquots of a 50%

suspension of these depleted cells in 0.15 M choline chloride or the incubation buffer (Krebs-Ringer buffer with 50 mM Hepes, pH 7.35) were added to flasks including 2.9 ml of the incubation buffer with or without a restorative agent such as 10 mM pyruvate, 20 mM ascorbate/0.1 mM phenazine methosulfate or 5 mM glucose, and were incubated for 5 min. Immediately after the incubation, the 30 s amino acid uptake was measured at 37°C by adding 20 μM labeled amino acid. Model amino acids used here were MeAIB for the (in the Ehrlich cell) main Na^+-dependent transport system, and BCH for the Na^+-independent transport system. Uptake of amino acid was terminated by pouring 10 ml ice-cold isotonic choline chloride/0.2% sucrose into the incubation flasks, followed immediately by a 2 min centrifugation at 1400 × *g*. The supernatant solution was then decanted, and using wicks of filter paper, the residual fluid removed from the cell pellets, which were weighed and extracted with 1 ml 3% sulfosalicyclic acid.

Aliquots of cell extracts and the supernatant solutions were assayed for radioactivity of the amino acid by scintillation counting [23]. Amino acid uptake has generally been presented as the ratio of apparent amino acid concentration in cell water to amino acid concentration in medium. Total water in the cell pellet was determined from the loss of weight during 12 h at 100°C. The extracellular water was estimated from dilution of sucrose added to the ice-cold choline chloride. Sucrose in the extracts and the supernatant solutions was determined by a spectrophotometric procedure [24]. In experiments in which deoxyglucose was added, extracellular water was determined instead from isotope dilution of [^{14}C]sucrose. Sodium and potassium in the extracts and the supernatant solutions were determined by use of a IL343 digital flame photometer (Instrumentation Laboratory Inc., Lexington, MA, U.S.A.).

Determination of ATP. After the restorative incubation, cells were centrifuged immediately for 2 min at 1400 × *g*. The supernatant was aspirated from the cell pellet. The pellet was extracted on ice with 2 ml of 0.4 N perchloric acid/0.05% disodium EDTA. After neutralizing the extract with 2.2 M $KHCO_3$, ATP was determined fluorometrically by measuring the reduction of $NADP^+$ in the presence of glucose, hexokinase and glucose-6-phosphate dehydrogenase [25]. When cells had been treated with phenazine methosulfate/ascorbate, cell extracts were mixed with 100 mg Dowex 50 W-X8 (H^+ form) in order to eliminate contamination by oxidized phenazine methosulfate, which otherwise oxidized NADPH non-enzymatically. After centrifugation of the mixture, 1 ml of the supernatant solution was then analyzed as usual for ATP.

Preparation of uniformly oriented plasma membrane vesicles. Plasma membrane vesicles from Ehrlich cells were prepared according to Kilberg and Christensen [26]. Those vesicles with an inside-out orientation were separated from other plasma membrane vesicles by lectin affinity chromotography: the membrane vesicle preparation was incubated with a concanavalin A-Sepharose 4B gel (Sigma, St. Louis, MO, U.S.A.) for 1 h at 4°C in the medium composed of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $MgCl_2$ and 5 mM KCl. After centrifugation at 50 × *g* the supernatant solution was removed and a second incubation with the gel was performed. The membrane pellet collected from the resulting supernatant by a subsequent centrifugation was shown to contain a vesicle population with 95—100% inside-out orientation. Vesicles with a right-

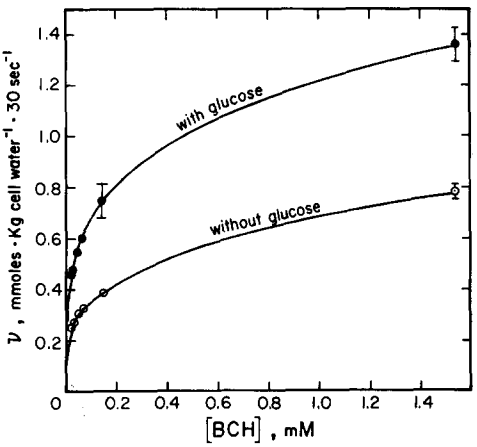
side-out orientation could be eluted from the resin with 0.1 M α-methylmannoside. Each preparation was monitored for orientation by assaying for accessible sialic acid and a marker enzyme in a fashion similar to that used by Steck and Kant [27]. The inside-out membrane vesicles were resuspended in the above-described Tris medium, and the 5 min uptake of 100 μM amino acids was measured. The right-side-out membrane vesicles were resuspended in 10 mM Tris-HCl buffer containing 100 mM NaCl or choline chloride, and the 30 s uptake of 100 μM amino acid was measured.

Assay for ATPase activity. The Mg^{2+}-ATPase activity was determined by incubating the isolated plasma membranes with 3 mM Tris-ATP in 50 mM Tris buffer, pH 7.4, containing 0.5 mM EGTA, 3 mM $MgCl_2$, and 1 mM mercaptoethanol. In the analysis for the $(Na^+ + K^+)$-ATPase, both 30 mM KCl and 100 mM NaCl were added to the incubation medium. The membranes and the inhibitors were incubated for 10 min at 37°C and the reaction initiated by adding Tris-ATP. The mixture (1 ml total volume) was incubated for 15 min at 37°C, after which 1 ml of cold 10% trichloroacetic acid was added to stop the reaction. Centrifugation resulted in a clear supernatant. Aliquots were removed for the determination of free inorganic phosphate by the method of Fiske and SubbaRow [28]. The Mg^{2+}-ATPase activity was taken to be the rate in the absence of added Na^+ or K^+, while the $(Na^+ + K^+)$-ATPase activity was calculated by the difference between the activities in the presence together and in the absence of the two alkali ions. Corrections were made for phosphate release from ATP in the absence of enzyme and from membranes in the absence of ATP. Protein determinations were by the Lowry method as modified by Markwell et al. [29].

Results

Concentrative uptake of the norbornane amino acid from Na^+-free medium greatly exceeds the simultaneously induced loss of endogenous amino acids

This behavior was observed for cells depleted in amino acids by the first procedure. The comparison was made both for cells that had subsequently been incubated 1 h in Krebs-Ringer bicarbonate/phosphate medium containing 5 mM glucose and for cells incubated in the same medium without glucose. Interestingly, the glucose treatment nearly doubled the 30 s uptake of BCH during the 60 min incubation along a sigmoid curve (data not shown), the initial rate also being nearly doubled (Fig. 1). Large stimuli by glucose were also seen when no phosphate was included in the medium, and during aeration with 95% oxygen/5% CO_2. Changes in cell volume were too small to account for the glucose effect. The 60 min presence of glucose in an N_2 atmosphere brought the subsequent 1 min uptake of BCH by amino acid-depleted cells to 9.4 ± 0.5 times the external level of 0.1 mM, which was ten times the intracellular level attained anaerobically without glucose, and equal to the value obtained in the presence of oxygen but no added energy source. These results extend to System L prior demonstrations that under anaerobic conditions glucose is highly stimulating to amino acid transport, and that oxygen is also stimulating in the absence of any added energy source. Fig. 2 shows for the glucose-treated cells comparisons of the uptake of BCH during 5 min with the simultaneously



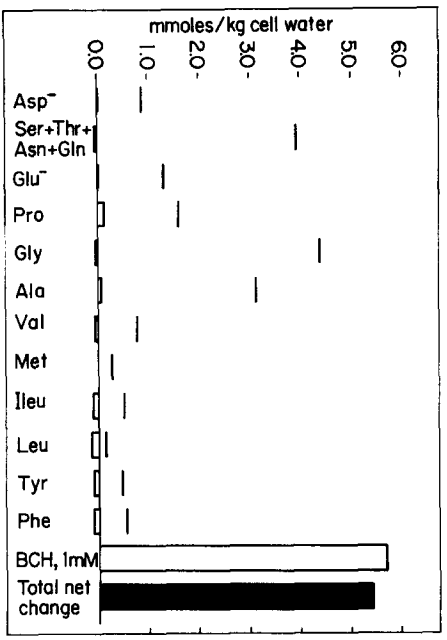


Fig. 1. Kinetics of BCH uptake into glucose-reenergized Ehrlich cells. Following depletion of endogenous amino acids the cells were either energized for 1 h with 5 mM glucose or incubated in the same medium containing no glucose. Energization was begun in 40 ml of modified Krebs-Ringer bicarbonate containing 10 mM phosphate and 15 mM bicarbonate (the energization is not sensitive to changes in phosphate concentration); each 10 min an additional 10-ml of substrate solution were added. We then observed the 30 s uptake of the b(—) isomer of BCH in the absence of sodium.

Fig. 2. Modifications in the intracellular pool of amino acids during 5 min induced by the presence of b(—) BCH. Simultaneous uptake of BCH. The Ehrlich cells were depleted of endogenous amino acids by the first method (Experimental procedure), and then incubated another hour in Krebs-Ringer bicarbonate/phosphate medium containing either no glucose or 5 mM glucose. Subsequently they were incubated for 5 min in the presence or the absence of [1-^{14}C]BCH. The cells were then analyzed for ^{14}C, and for various amino acids chromatographically, using the automatic amino acid analyzer. The vertical lines indicate the levels to which the various amino acids had been brought by the depletion and the restoration with glucose. The short bars on the ordinate show the net change induced for each amino acid by the 5 min presence of BCH. The longest bar shows the cellular gain in BCH, whereas the solid bar at the bottom shows the portion of the BCH uptake not accounted for by concurrent induced net exodus of cellular amino acids.

induced loss of cellular amino acids, i.e. with the difference between the residual levels of each amino acid (as recorded by the automated amino acid analyzer) with and without 1 mM BCH present during the 5 min. The amounts of BCH taken up (mmol) were in general over ten times the net induced loss of cellular amino acids. When no glucose was provided during the 60 min incubation, a similar behavior was seen.

The cells treated 60 min with glucose were not significantly inhibited in their 5 min uptake of BCH by the presence of 0.2 mM dinitrophenol, 2 mM cyanide, 100 mM sodium azide, 5 μM oligomycin, 1 μM valinomycin or gramicidin, or 0.04 mg/l colchimide, nor stimulated by 0.1 mM phenazine methosulfate plus 20 mM ascorbate nor 1 mM cyclic AMP. Uptake was, however, diminished to about one-seventh by the simultaneous presence of 2 mM quinacrine, a sensitivity previously shown characteristic for the NADH dehydrogenase activity

of the plasma membrane [26,30], and applicable to the restoration of amino acid transport after poisoning with dinitrophenol plus iodoacetate [20].

Effects of external Na^+ and K^+ on restoration of amino acid uptake

Fig. 3 shows that increments of Na^+ in the suspending medium did not enhance the restoration of the uptake of BCH produced by pyruvate or by phenazine methosulfate/ascorbate after energy depletion by the first method. In correspondence, its restoration did not depend on the calculated Na^+ gradient across the plasma membrane (data not shown). Even in almost Na^+-free medium uptake was restored by the addition of pyruvate or phenazine methosulfate/ascorbate. Fig. 4 shows that the proportion of MeAIB uptake restored by pyruvate or phenazine methosulfate/ascorbate did not increase with external Na^+. At 1 mM Na^+ the rates of uptake were so low that the comparison should not be considered valid. Table I shows that restoration of BCH uptake was also obtained in potassium-free medium, although MeAIB uptake was not well restored in potassium-free medium in spite of elevated ATP levels.

A similar distinction between the restoration of uptake of BCH and of MeAIB was observed in the different sensitivity to the inhibitory effect of ouabain (Fig. 5). This agent at 2 mM completely prevented the restoration of MeAIB uptake induced by pyruvate, whereas it approximately halved the restoration of BCH uptake.

Question of requirement of ATP production for the restoration of BCH uptake

As shown in Table I, the restoration of amino acid uptake was accompanied by an increase in cellular ATP levels. With a corrected analytical procedure described under Experimental procedure phenazine methosulfate/ascorbate was

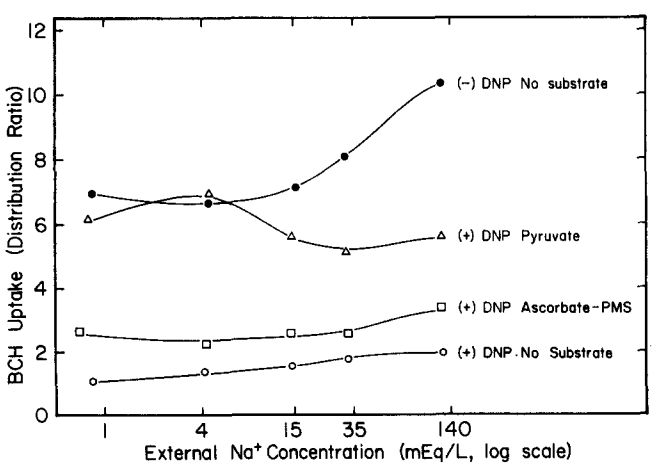


Fig. 3. Effect of external Na^+ concentration on the restoration of BCH uptake induced by pyruvate or ascorbate/phenazine methosulfate. After 30 min incubation with 0.2 mM dinitrophenol (DNP), cells were added to 10 mM pyruvate or 20 mM ascorbate/0.1 mM PMS and incubated 5 min. The 30 s BCH uptake was then measured as described in Experimental procedure. Hepes/NaOH in the incubation medium was partly replaced by Hepes/choline hydroxide. External Na^+ concentration represents the average of Na^+ concentrations in the medium before and after the incubation. —DNP, without DNP treatment; +DNP, with DNP treatment. PMS, phenazine methosulfate.

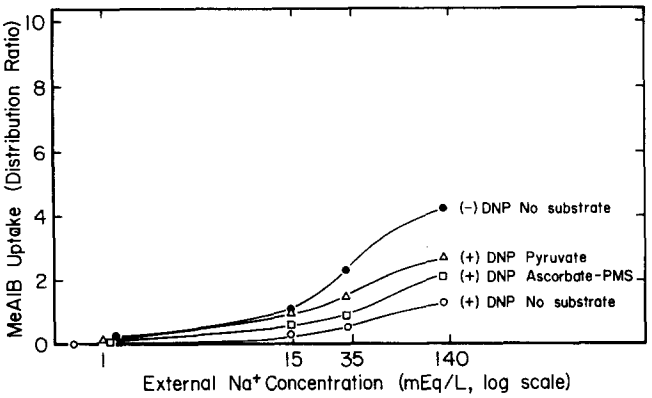


Fig. 4. Effect of external Na^+ concentration on the restoration of MeAIB uptake induced by pyruvate or ascorbate/phenazine methosulfate. For experimental details, see Fig. 3.

shown to cause an early increase in cellular ATP levels, which had been masked in our earlier experiments [12]. In order to determine whether or not ATP production is necessary for the restoration of amino acid uptake, various metabolic inhibitors of ATP production were provided simultaneously for 5 min with

TABLE I

EFFECT OF EXTERNAL POTASSIUM CONCENTRATION ON RESTORATION OF MeAIB OR BCH UPTAKE INDUCED BY ASCORBATE/PHENAZINE METHOSULFATE OR SODIUM PYRUVATE IN EHRLICH CELLS

Cells were incubated in a 50-fold volume of medium with or without K^+ for 5 min. Amino acid uptake was then determined by 30-s uptake of 20 μM MeAIB or BCH. DNP, 2,4-dinitrophenol.

Test amino acid	Treatment	Initial medium K^+ (mequiv./l)	Amino acid uptake (distribution ratio)	$[ATP]_{cell}$ (mM)	Combined alkali ion gradients *
MeAIB	Before 0.2 mM DNP	0	2.42	1.85	>500
		5	7.26	1.85	88.5
	After 0.2 mM DNP				
	5 min restoration	0	1.42	0.15	>500
	with no substrate	5	1.43	0.12	27.7
	20 mM ascorbate/	0	1.76	0.54	>500
	0.1 mM phenazine methosulfate	5	3.70	0.46	32.9
	10 mM pyruvate	0	1.41	1.14	>500
		5	4.01	1.03	35.5
BCH	Before 0.2 mM DNP	0	14.16	2.20	>500
		5	16.39	2.22	85.7
	After 0.2 mM DNP				
	5 min restoration	0	1.78	0.07	>500
	with no substrate	5	1.99	0.07	27.2
	20 mM ascorbate/	0	2.94	0.47	>500
	0.1 mM phenazine methosulfate	5	2.98	0.36	32.8
	10 mM pyruvate	0	5.05	0.94	>500
		5	5.50	1.03	28.6

* $[Na^+]_{out} \cdot [K^+]_{in}/[Na^+]_{in} \cdot [K^+]_{out}$.

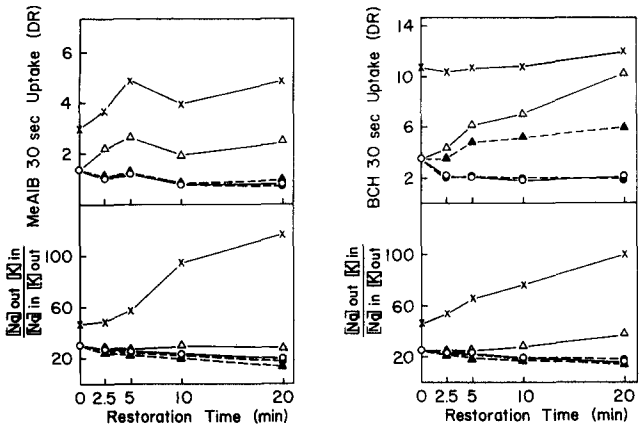


Fig. 5. Time course for the restoration of uptake of amino acids induced by pyruvate and effect of ouabain on it. Upper figures show the distribution ratios (DR) of amino acid reached in 30 s, and lower figures show combined alkali-ion gradient ($[Na^+]_{out} \times [K^+]_{in}/[Na^+]_{in} \times [K^+]_{out}$). Pyruvate and/or ouabain (2 mM) were added simultaneously after a 30 min incubation with dinitrophenol. X, with neither dinitrophenol treatment nor pyruvate; ○, without pyruvate after dinitrophenol treatment; ●, with ouabain only after dinitrophenol treatment; △, with pyruvate after dinitrophenol treatment; ▲, with pyruvate and ouabain after dinitrophenol treatment.

each of the restorative agents. Fig. 6 compares the influences of those metabolic inhibitors on restoration of BCH uptake and of ATP levels induced by pyruvate, phenazine methosulfate/ascorbate or glucose. Restoration of BCH uptake may by noted on the left side of the figure. Dinitrophenol and oligomycin almost completely prevented the restoration by pyruvate or phenazine methosulfate/ascorbate. Rotenone completely eliminated the restoration of BCH uptake by pyruvate, but not that by phenazine methosulfate/ascorbate. Conversely, sodium cyanide prevented the restoration by phenazine methosulfate/ascorbate, but not that by pyruvate, presumably because cyanide is soon destroyed by pyruvate. Iodoacetate, an inhibitor of glycolysis, did not significantly affect the restoration by either agent, although as predicted, it did inhibit the restoration by glucose. The restoration of BCH uptake by glucose was even stimulated by mitochondrial inhibitors such as dinitrophenol and rotenone. 30 μM antimycin A had an effect substantially similar to that of rotenone on the restoration of BCH uptake (data not shown).

Generally good correspondence was observed, moreover, between changes in BCH uptake and cellular ATP levels modified by metabolic inhibitors except for certain conditions including deoxyglucose addition. Deoxyglucose decreased the restoration of BCH uptake induced by phenazine methosulfate/ascorbate, but it had little effect in preventing the ATP rise. In a repeat test by G.K., however, this decrease by deoxyglucose was not confirmed, although restoration by glucose was inhibited. Deoxyglucose is known to decrease ATP levels by inducing phosphorylation of this sugar. These results show that ATP production either in mitochondria or cytoplasm is usually associated with the restoration of amino acid uptake, but that the association between ATP production and the energization of amino acid uptake at the plasma membrane is imperfect and perhaps not direct. This conclusion was supported by the next three experiments.

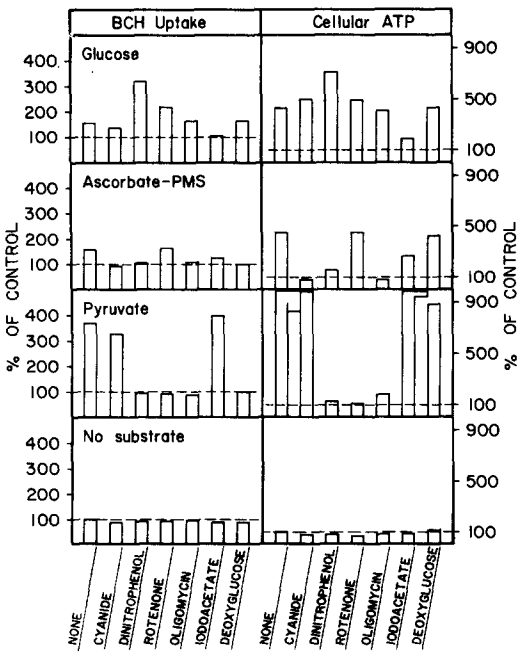


Fig. 6. Comparison of the influence of a series of metabolic inhibitors of ATP production on the restoration of BCH uptake and of ATP levels induced by pyruvate, ascorbate/phenazine methosulfate or glucose. Each bar represents percentage of uptake or of the ATP levels for controls with neither a restorative substrate nor an inhibitor (the mean of values from two or three experiments). Concentrations of inhibitors used here were: $2 \cdot 10^{-3}$ M sodium cyanide; $2 \cdot 10^{-4}$ M dinitrophenol; $5 \cdot 10^{-7}$ M rotenone; $5 \cdot 10^{-6}$ M oligomycin; $1 \cdot 10^{-3}$ M iodoacetate; $5 \cdot 10^{-3}$ M 2-deoxy-D-glucose.

Indirect participation of ATP in the energization of amino acid transport

First, when Ehrlich cells were incubated with 66 μM quercetin, an inhibitor of both $(Na^+ + K^+)$-ATPase and mitochondrial ATPase [31,32], for 30 min before treatment with pyruvate, this agent did not prevent the restoration of uptake of two model amino acids, but on the contrary tended to improve the restoration and the ATP levels in approximate coordination (Table II). This table also shows that quercetin is a very effective inhibitor of the $(Na^+ + K^+)$-ATPase of the Ehrlich cell. Hence its failure to inhibit restoration shows that even though ATP may be involved in restoration, the $(Na^+ + K^+)$-ATPase is not. If ATP acts through an ATPase, it must be through another such enzyme: the so-called Mg^{2+}-ATPase activity is not significantly influenced by quercetin.

Second, 5—400 μM atractyloside, an inhibitor of the adenine nucleotide 'translocase' of mitochondria [33] had little effect on the restoration of BCH uptake induced by pyruvate, when it was added for 10 min before pyruvate was added (data not shown). Taking into account its intense toxicity, one would expect this agent to have access to and to interfere with the delivery of mitochondrial ATP to the plasma membrane in the intact cell.

Finally and more directly, when 3 mM ATP was added to the inside-out membrane vesicles, the exodus of BCH from the membrane vesicles was not significantly enhanced (exodus in vesicles treated with ATP was only 4.4% higher than that in controls). The exodus of amino acid from the inside-out

TABLE II

EFFECT OF QUERCETIN TREATMENT ON PYRUVATE RESTORATION OF AMINO ACID UPTAKE: COMPARISON OF EFFECT OF OUABAIN AND QUERCETIN ON ATPase ACTIVITIES

Quercetin was dissolved in ethanol/Me_2SO (2 : 3), and an equal volume of solvent added to the control assays. For the uptake experiments (above) the cells were depleted of energy by an incubation at 37°C for 30 min with 2,4-dinitrophenol (DNP) (second depletion method). Following a 5 min incubation in the presence of 10 mM pyruvate, amino acid uptake (30 s) was tested. The quercetin was present during the entire 35 min incubation period. The agents were added to the vesicular membrane preparation (below) for 10 min at 37°C prior to the assays for ATPase activity.

Agent	Percent of control rate				
	MeAIB uptake	BCH uptake	Cellular ATP	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase
None	100	100	100		
66 μM quercetin	96	103	21		
10 mM pyruvate	317	362	1020		
66 μM quercetin and 10 mM pyruvate	345	400	1290		
None				100 *	100 **
1 mM ouabain				104	9
80 μM quercetin				86	38

* 4.40 ± 0.3 μmol P_i released/h per mg protein.
** 21.7 μmol P_i released/h per mg protein.

membrane vesicles may be considered to correspond functionally to amino acid uptake into whole cells. This behavior suggests then that ATP alone may fail to link directly to activate amino acid transport in the plasma membrane. In contrast to ATP, as shown in Table III both NADH and phenazine methosulfate/ascorbate significantly enhance the exodus of both model amino acids from inside-out plasma membrane vesicles. Furthermore, phenazine methosulfate/ascorbate stimulated the 30 s uptake of MeAIB in right-side-out membrane vesicles (data not shown). Because a high activity of an NADH dehydrogenase is found in the plasma membrane of Ehrlich cells [12,26,34], these results indicate that some reducing compound such as NADH may be involved as an energy source alternative to ATP in a mode of energization of amino acid transport by the plasma membrane.

TABLE III

INFLUENCE OF ELECTRON DONORS ON THE EXODUS OF AMINO ACIDS FROM INSIDE-OUT PLASMA MEMBRANE VESICLES

See the text in Experimental procedure. Each value represents the mean ± S.D. (n = 3).

Additions	MeAIB		BCH	
	pmol uptake/ mg protein	% change from control	pmol uptake/ mg protein	% change from control
None	163.8 ± 5.7	—	516.8 ± 20.0	—
NADH (1 mM)	128.8 ± 8.2	—21.4	442.7 ± 46.7	—14.3
Phenazine methosulfate/ascorbate (0.2—20.0 mM)	113.2 ± 6.1	—30.9	351.1 ± 19.8	—32.1

In Ehrlich cells NADH reducing equivalents are known to be transferred from mitochondria to cytoplasm by the malate-aspartate shuttle [35]. A contribution of this shuttle to the energization of amino acid transport should be recognizable by a sensitivity to inhibition by aminooxyacetate, a pyridoxal phosphate-binding reagent [36,37], or by fluoromalate, an inhibitor of malate dehydrogenase and the malate carrier [38]. Neither 0.5 mM aminooxyacetate nor 2 mM fluoromalate added 30 min before pyruvate, however, significantly inhibited the restoration of uptake of MeAIB or BCH induced by pyruvate (data not shown).

Discussion

The foregoing experiments reemphasize the net uphill operation of Na^+-independent System L, its response to glucose and other energy sources, and the necessity of taking this system into account, along with the Na^+-dependent systems, in efforts to explain transport energization.

If the restorative action of pyruvate or phenazine methosulfate/ascorbate were produced by its increasing the portion of the electrochemical gradient of Na^+ that we can arbitrarily assign to the transmembrane difference in Na^+ concentration, or that due to the transmembrane potential, then we should expect increments in external Na^+ concentration present during the 30 s interval of uptake to enhance the restoration of uphill transport. Figs. 3 and 4 fail to show any such enhancement. The difference in the sensitivity of the restoration of uptake of the two test amino acids to the presence of extracellular K^+ (Table I) and ouabain (Fig. 5) appear to be characteristic of the Na^+-dependent and the Na^+-independent systems, respectively, in that these two agents influence the maintenance of electrogenic Na^+/K^+ pumping [6,7]. Extracellular K^+ can also serve to control respiration [39]. Under the conditions used here, however, we found no significant difference in the cellular ATP levels between cells in normal medium and in K^+-free medium (Table I), indicating that a lack of extracellular K^+ had not disturbed respiration. Hence external K^+ may be needed for the Na^+-dependent transport of MeAIB at the plasma membrane, rather than for energization in any central way. Similarly, ouabain blocked only partially the alkali ion-independent restoration of BCH uptake, while completely blocking the restoration of MeAIB uptake [12]. Moreover, uptake of MeAIB into right-side-out vesicles was inhibited by 1 mM ouabain, even though the medium contained no K^+ and an artificial inwardly directed Na^+ gradient was imposed. Under these conditions the inhibitory action of ouabain should not be due to its inhibition of $(Na^+ + K^+)$-ATPase activity, as others have pointed out.

ATP production was usually associated with the restoration of amino acid uptake (Fig. 6). Restoration of BCH uptake and of ATP levels induced by pyruvate was prevented by all the respiratory poisons tested except cyanide, whereas restoration by phenazine methosulfate/ascorbate was not blocked by either rotenone or antimycin, but by cyanide. Phenazine methosulfate/ascorbate acts as a selective electron donor system to site III of the respiratory chain [40] as well as a donor to redox activity of the plasma membrane. In contrast to restoration by either pyruvate or phenazine methosulfate/ascorbate, restoration by glucose was blocked by iodoacetate and even stimulated by rotenone,

antimycin, or dinitrophenol. These findings indicate that pyruvate and phenazine methosulfate/ascorbate energize amino acid transport by a pathway linked to mitochondrial ATP production, whereas glucose does so mainly by a pathway linked to cytoplasmic ATP production, even though the restoration by glucose can be modified by disturbance of mitochondrial function, presumably through the Crabtree effect. Because phenazine methosulfate/ascorbate enhances amino acid transport by plasma membrane vesicles from which mitochondria are absent (Table III), this restorative agent may possibly energize amino acid transport by action at the plasma membrane, as well as by stimulation of mitochondrial ATP production.

Although ATP still needs to be considered further as a primary transfer form of metabolic energy for uphill amino acid transport, the present work indicates that this substance may be involved only indirectly in the energization mechanism for the transport, in part because ATP fails to increase the exodus of BCH from inside-out membrane vesicles. It remains possible, of course, that a relevant ATPase activity so far undetected has undergone alteration during separation of the membrane vesicles. The failure of quercetin to prevent the restoration for both model amino acids indicates that the $(Na^+ + K^+)$-ATPase hardly contributes to the restoration, although the test does not exclude an unknown quercetin-insensitive ATPase. Quercetin and ouabain are thought to act on the same substance, namely the $(Na^+ + K^+)$-ATPase; yet ouabain inhibits pyruvate restoration of transport whether Na^+ dependent or Na^+ independent, whereas quercetin actually potentiates restoration by pyruvate. Clearly the two act in different ways. In contrast to the inactivity of ATP, NADH and phenazine methosulfate/ascorbate stimulated amino acid exodus from the inside-out vesicles, in the apparent absence of ATP (Table III). The small effect of atractyloside on the restoration of BCH uptake and ATP levels induced by pyruvate suggests that channeling of ATP from mitochondria to cytoplasm may not be a critical step for the participation of ATP in the amino acid transport energization.

In previous reports [12,13,26] we proposed that an oxidoreduction activity in the plasma membrane, including an NADH dehydrogenase, may participate in supplying energy both to Na^+-dependent and to Na^+-independent amino acid transport, by allowing utilization of reducing equivalents perhaps made available by a shuttle from mitochondria, but which could also arise in the cytoplasm. Enzyme systems of this type and location continue to receive attention (see review Ref. 41, and for examples Refs. 26, 42 and 43). The participation of an NADH dehydrogenase in amino acid transport across the plasma membrane of the Ehrlich cell receives support from the NADH stimulation of amino acid efflux from the inside-out vesicles (Table III). Added pyruvate has also been reported to control availability of cytoplasmic NADPH in Ehrlich cells [44,45]. In an abstract, Kawasaki and Yamamoto [46] report confirmation of our evidence for the role of plasma membrane redox activity in the Na^+-dependent transport of 2-aminoisobutyric acid.

The bidirectional malate-aspartate shuttle which transfers the reducing equivalents of NADH across the mitochondrial membrane is known to serve in Ehrlich cells [33,47,48]. Inhibitors of this shuttle proved, however, to have little effect on the pyruvate-induced restoration of uptake of either model

amino acid. Other possible shuttles should be investigated.

The mitochondrial product presumed to energize the plasma membrane might be reducing equivalents of NADH whose production and export may have a necessary association with ATP production. Energy proposed to arise via the NADH dehydrogenase activity, using reducing equivalents of either mitochondrial or cytoplasmic NADH, may be supplementary or alternative to an energization by ATP itself and supplementary to energy supplied to System L by outflow of cellular amino acids, and to the Na^+-dependent systems through cotransport with Na^+. The energization serving for System L appears not to act by providing a transmembrane gradient of H^+ or of the electrical potential [50]. If the particle presumably subjected to an initial charge separation is smaller than the amino acid molecule, any energy-transferring gradient of that particle must be largely hidden within the substance of the membrane [49].

Acknowledgements

The work was supported by a research grant (HD01233), and as a pilot project under the Michigan Diabetes Research and Training Center (Grant IP60 AM20572), both from the National Institutes of Health, U.S. Public Health Service.

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