Biochimica et Biophysica Acta, 356 (1974) 319–330

BBA 76693

ROLE OF ATP ON THE INITIAL RATE OF AMINO ACID UPTAKE IN EHRLICH ASCITES CELLS

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(Received February 26th, 1974)

SUMMARY

Incubation with graded doses of rotenone can bring about a graded lowering of the cellular ATP level. Using cells with varying ATP levels, it can be shown that the initial uptake of [^{14}C]glycine, before the cellular concentration exceeds that of the medium, is decreased as ATP decreases. Alterations in cellular cations cannot account for the difference in glycine influx. Prolonged exposure of cells to a lowered ATP content increases the exodus of cellular glycine. Valinomycin reduces the steady-state level of glycine uptake, but its effects can to a major extent be overcome by the addition of glucose. At high extracellular K^+ (70 mM) neither the sum of the $Na^+ + K^+$ gradients, nor the electrochemical potential of Na^+ provides sufficient energy to account for glycine and 2-aminoisobutyrate accumulation if a 1 : 1 coupling between Na^+ and the amino acid occurs.

INTRODUCTION

In recent years there has been considerable discussion about the nature of the energy used in Na^+-coupled, non-electrolyte transport to attain accumulation of non-electrolytes against an electro-chemical gradient. Several possibilities have been considered and each has gained supporters (see Symposium on coupled solute transport [1]).

The concept that cation gradients may provide energy for accumulation was first suggested by Christensen and Riggs [2] and subsequently developed into a "Na^+ gradient hypothesis" by Crane [3] and by Schultz and Curran [4]. The basic assumption in this hypothesis is that the carrier catalyzes an reversibel reaction whose asymmetric activity is maintained by the asymmetric distribution of Na^+ and K^+. Reversal of the gradients should show reversed uphill flow of organic solute. Reports that such reversal of flow does occur have been made [5–9]. Noteworthy, however, are the facts that to demonstrate reversal, the experiments must be done in Na^+-free medium and that the degree of reversibility is small [5–9].

Several authors have questioned the validity of this hypothesis because ac-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

cumulation of organic solutes against their respective electrochemical potentials occurs despite absence of a Na^+ gradient and even in face of reversed Na^+ and K^+ gradients [9–15].

While some investigators consider ion gradients the only energy source [16] others have suggested that part of the energy for accumulation may also be derived from metabolic energy [10] or from the transmembrane potential [17].

The fact that Ehrlich ascites cells containing ATP generally show higher rates of transport and greater accumulation than cells in absence of ATP, has led Gibb and Eddy [17] to propose that either an increased transmembrane potential or better coupling to a K^+ gradient provide additional energy when ATP is available.

Other investigators in this area have tended to minimize the contribution of cation gradients as potential sources of energy and have concluded that ATP (or some such compound) provides energy and that gradients do not provide a major ($> 10\,\%$) energy component in the transport of organic solutes [9, 19–23].

According to the early proposals of the gradient hypothesis [3, 4], influx of a neutral organic solute down its own electrochemical gradient will not be affected by the magnitude of the cation gradients or the level of cellular ATP, but the net flux will be markedly affected since the efflux will increase as intracellular Na^+ increases. Therefore an experimental approach to test this hypothesis is to examine influx as a function of cellular ATP concentration and at fixed cation gradients before accumulation against a gradient occurs. Dependance of influx on the ATP level would be inconsistent with the predictions of the ion gradient hypothesis and would show evidence for a direct role for ATP in transport of organic solutes. We have attempted a series of experiments of this type and have been able to show that prior to significant accumulation, influx of glycine at equivalent ion gradients is a function of cellular ATP concentration. In addition our studies have yielded data which suggest that on thermodynamic considerations, it is unlikely that the electrochemical potential for Na^+ is the major energy source for amino acid accumulation.

MATERIALS AND METHODS

Ehrlich ascites cells were maintained and harvested as previously described [9]. Incubations were carried out in a Krebs–Ringer medium buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) which contained 150 mM NaCl, 15 mM K^+, 1.45 mM Mg^{2+}. When NaCl was used at 100 mM one third of the NaCl was replaced by KCl. Incubations were carried out in beakers, in a shaking water bath at 37 °C.

The washed cell suspension (1/20 dilution) was added to the incubation medium which had been brought to the temperature of the bath. The final ratio of cells to medium was 1/60 so that a 2-ml sample yielded a fresh weight of tissue of 35 ± 5 mg.

Except for efflux experiments, uptake was initiated by the addition of the ^{14}C-labelled amino acid. Samples (2 ml volume) were rapidly added to 4 times their volume of chilled isotonic Ringer solution in tared centrifuge tubes and centrifuged for 2 min at $1400 \times g$. The medium was decanted, the tubes recentrifuged and the fluid remaining dried with lint-free tissue paper. Amino acids were extracted with 95 % ethanol and an aliquot counted in a liquid scintillation counter using a modified

Bray's solvent [24]. Prior to extraction with ethanol, the cell pellet was weighed to obtain fresh weight. After ethanol extraction, the tissue residue was dried to constant weight to obtain the dry weight. The dry weight was 13–15 % of the fresh weight.

For ion determinations, the cells were washed with isotonic choline chloride and digested with HNO_3. Na^+ and K^+ were determined by flame photometry. ATP was determined by the luciferase method as described by Stanley and Williams [25] using a Packard scintillation counter.

Extracellular volume was determined in separate experiments with [^{14}C]-inulin and was found to be 30–35 % of the fresh weight of the pellet.

Valinomycin was purchased from CalBiochem, San Diego, Calif. Rotenone was a product of Sigma Chemical Co., St. Louis, Mo.

All radioisotopes were obtained from New England Nuclear, Boston, Mass.

RESULTS

To examine the effect of ATP on the rate of amino acid uptake, we required an inhibitor which would reduce ATP to predictable levels and, once attained, these levels could be maintained constant for the duration of the experimental period (about 15 min). We have found that rotenone in the range between 0.1 and 16 ng/ml was satisfactory in this respect, and that about 15–20 min incubation at 37 °C was required to achieve a new steady-state level of ATP. We observed that with lower Na^+ and higher K^+ in the incubation medium, more rotenone (about double the amount) is required to decrease the ATP level to the same extent (results not shown). Presumably this is due to the fact that with 100 mM Na^+ and 65 mM K^+ there is less activation of the Na^+ pump and hence less ATP utilization. The data in Table I show that, once achieved, the lowered ATP levels remain constant for the duration of the experimental period.

Glycine uptake was measured at 1-min intervals over a period of 4 min. With 1 mM glycine, uptake was linear with time for the first 3 min of incubation but the curve did not pass through the origin. A "residual" uptake of about 0.2 μmole/ml cell water persisted with 1 mM glycine in the medium; this component was equal to the uptake in a choline medium at 37 °C in presence of excess methionine (to suppress glycine transport [26]). Moreover, if the incubation in choline is carried out at 0 °C an uptake of 0.22 μmole/ml cell water was observed near steady state (30 min). Thus there appears to be some component in the uptake, which is either mediated by a separate transport mechanism or is due to diffusion which distorts early values for uptake, particularly uptakes at 1 min or earlier. Therefore all subsequent figures and tables have been corrected for this baseline uptake. The data in Table I from two representative experiments show the rate of glycine uptake at varying cellular ATP levels with two types of incubation medium. The average Na^+ and K^+ gradients during the experimental period are also given. The initial rate of glycine uptake increases as the ATP level increases, although there is little accumulation above the medium concentration. We have expressed our results in terms of the cellular [^{14}C]glycine concentration to highlight this observation. Under the experimental conditions used, the ratio of cell water to dry weight is constant, therefore the data may be expressed per mg dry weight with no change in the relative results. (3.7 μl cellular water are present per mg dry wt of tissue [9].)

TABLE I

AMINO ACID UPTAKE, ATP LEVELS AND CATION DISTRIBUTIONS

Glycine uptake from a 1 mM [^{14}C]glycine solution (spec. act. 300 cpm/nmole) was measured at intervals from 60 s to 3 min. The average rate per min is given after correction for non-specific uptake. Samples for cations and ATP levels were taken from duplicate flasks just before and 15 min after initiation of glycine uptake measurements. Incubation was at 37 °C at pH 7.4. The ATP level was varied by preincubation with different concentrations of rotenone from 0.8 to 16 ng/ml incubation medium. The control values are those without rotenone. The preincubation with and without rotenone was carried out for 30 min at 37 °C before adding ^{14}C-labelled amino acid to measure uptake.

Expt No.	Conditions	Uptake of glycine μmoles/ml cell water·min	ATP concn (mM)		Cellular cations (mM)	
			t_0	t_{15}	Average Na_i^+	Average K_i^+
I	Normal Ringer	0.50	0.30	0.40	163	55
		1.20	1.70	1.50	119	117
		1.50	2.20	1.80	97	129
	Control	1.70	2.50	—	90	150
II	100 mM Na^+ plus 65 mM K^+	0.40	0.23	0.13	82	91
		0.52	0.40	0.51	73	95
		0.57	1.10	0.90	72	106
		0.67	1.50	1.60	56	116
	Control	0.82	1.70	—	48	124

It is evident from Table I that we have not been able to eliminate completely changes in the Na^+ gradient while varying cellular ATP concentration. These changes in the Na^+ gradient make interpretation more difficult.

Although Chez et al. [27] had concluded that the magnitude of the Na^+ gradient did not influence alanine influx as opposed to net flux in rabbit ileum, recent reports have suggested that the influx of an organic solute may be influenced by the magnitude of the Na^+ gradient [28, 29]. In view of these conclusions and the data in Table I, we designed experiments to test whether the change in influx is due primarily to the changes in cation distributions or to cellular ATP levels. Two types of experiments were tried. (1) Cells were first incubated in choline medium with and without rotenone (Na^+-free, normal K^+). Then the cells were transferred to medium with 100 mM Na^++65 mM K^+. In this situation, the initial rate of uptake in cells with ion gradients of nearly equal magnitude but different ATP levels can be compared. (2) Cells were first incubated with rotenone but in media of different composition so that different cellular cation levels would be obtained. Upon introducing the cells into normal Ringer medium, different cation gradients would be obtained. One aliquot of cells was incubated in the absence of rotenone but in an all Na^+ medium resulting in a cell with high ATP and high Na_i^+. In this manner we obtained data on the relative contribution of the cation gradients and ATP on the initial rate of glycine uptake.

The data in Fig. 1 show that despite nearly equivalent cation distributions there is a marked difference in the initial rate of glycine uptake which is in line with the differences in the ATP levels. Data from two representative experiments are shown. In Fig. 2 it is evident that cells with high cellular Na^+ and ATP show higher rates of transport than cells with low cellular Na^+ and low ATP.

ATP and exodus of amino acids

One of the observations we made in the course of these experiments was that the $t_{\frac{1}{2}}$ for steady state was reduced in cells with decreasing ATP levels. The implication of this observation appeared to be that the cellular ATP level might influence the efflux of cellular amino acids.

Earlier studies had indicated that addition of 2,4-dinitrophenol did not alter the rate of loss of cellular amino acid but did alter the uptake within 5–10 min of its addition [26, 30, 31]. It is conceivable that earlier experiments aimed to test this possibility were negative only because measurements of amino acid efflux were carried out before sufficient time elapsed to allow the ATP level to decrease. In the present experiments cells were brought to a lower level of ATP before examination of amino acid exodus. The results show (Fig. 3) that at low levels of cellular ATP, the $t_{\frac{1}{2}}$ for exodus is shortened; that is the rate of loss is enhanced. If rotenone is added at the start of measurements of exit, there is little observable change in rate of loss of cellular glycine, an observation consistent with the original observations [26, 30, 31].

Since the accumulation of amino acid is greatly decreased in presence of rotenone, we examined the effect of cellular glycine concentration on efflux in presence of rotenone. No difference in $t_{\frac{1}{2}}$ was obtained for a 10-fold increase in cellular glycine concentration (results not shown).

In the present experiments, it is considered unlikely that the differences in the rate of exit are due to differences in "recapture" of released amino acid [31] for the

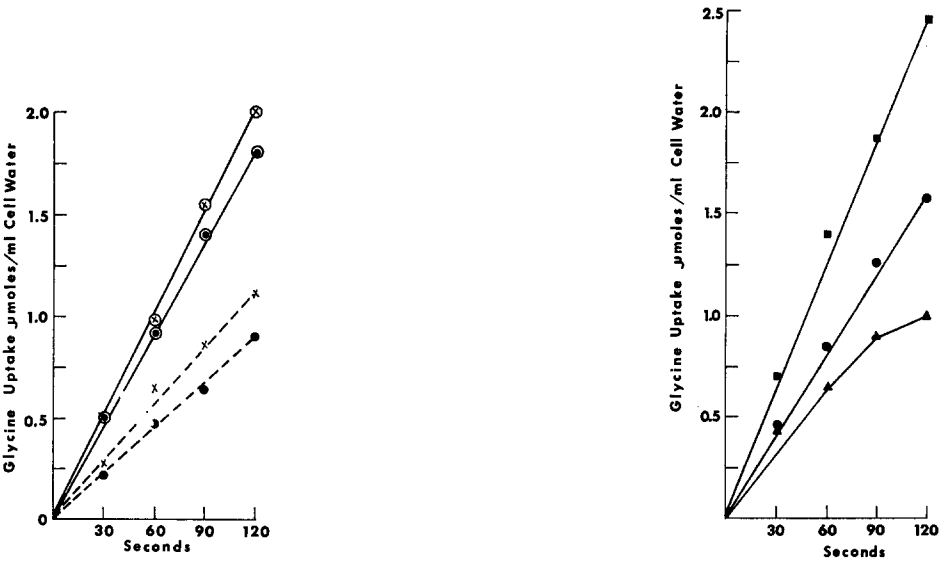

Fig. 1. Effect of preincubation in choline chloride with and without rotenone on the initial rate of glycine uptake.

	ATP concn (mM)		Cellular cations (mM)			
	t_0	t_5	$Na_{t_0}^+$	$Na_{t_5}^+$	$K_{t_0}^+$	$K_{t_5}^+$
Control (A); ⊙–⊙)	1.02	1.05	41	39	91	84
+rotenone (A); ●–●)	0.066	0.051	66	52	74	68
Control (B; ⊗–⊗)	1.0	1.4	31	31	81	104
+rotenone (B; ×–×)	0.13	0.065	44	53	81	79

Cells were preincubated for 30 min in isotonic choline chloride with 16 ng/ml of rotenone (●, ×) and without (⊙, ⊗) rotenone. Then the cells were centrifuged and transferred to fresh isotonic 100 mM Na^++65 mM K^+ medium containing the same concentration of rotenone as in the preincubation. HEPES buffer was used. Incubation throughout was at 37 °C. Glycine uptake from a 1 mM [^{14}C]glycine solution was measured at the times given. Results from two typical (A, (⊙, ●); B, (⊗, ×)) experiments are given. Corrections for non-specific uptake have been made. The ATP values and ion distributions for Expts A and B are given above.

Fig. 2. Relative effects of low Na_i^+ and ATP on initial rate of glycine uptake. Cells were preincubated with rotenone in a choline chloride medium and in an all Na^+ medium, and without rotenone in all Na medium (150 Na^+, <1.0 mM K^+) for 30 min. The cells were centrifuged and preincubation medium removed as completely as possible. Then the cells were transferred to normal Ringer medium (150 Na^+, 15 K^+), and uptake of glycine was measured from a 1 mM solution. All incubations were at 37 °C in a HEPES buffer (pH 7.4). Samples for cellular cations and ATP were taken from duplicate flasks at 2 min before and 2 min after glycine uptake. ●, preincubation in choline chloride+rotenone; ▲, preincubation in NaCl+rotenone; ■, preincubation in NaCl without rotenone. Corrections for non-specific uptake have been made. The ATP levels and cation distributions at t = +2 and t = −2 min are given below. The results presented are from a typical experiment.

	ATP concn (mM)		Cellular cations (mM)			
	t_{-2}	t_{+2}	Na_{t-2}^+	Na_{t+2}^+	K_{t-2}^+	K_{t+2}^+
+rotenone (●–●)	0.12	0.05	50	53	83	72
+rotenone (▲–▲)	0.08	0.04	124	131	55	48
−rotenone (■–■)	1.2	1.6	114	62	98	126

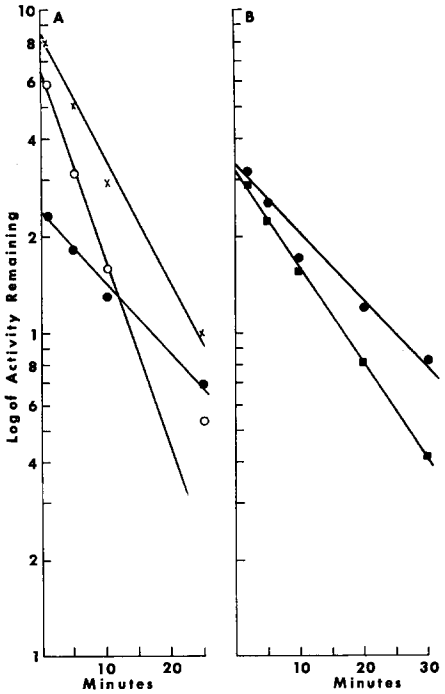

Fig. 3. Efflux of [1-^{14}C]glycine in cells preincubated with and without rotenone. Cells were preincubated with 1 mM [^{14}C]glycine ± rotenone for 40 min. Rotenone was added to the cells 15 min before glycine. After the preincubation, cells were centrifuged and the supernatant medium removed as completely as possible. The cells were then resuspended in 1 ml of Krebs–Ringer medium and added to fresh medium, free from amino acids but with the same concentration of rotenone as in the preincubation. Samples were withdrawn and centrifuged without washing. ×–×, preincubation with 4 ng/ml rotenone; ○–○, preincubation with 16 ng/ml rotenone; ●–●, control; ■–■, rotenone added during efflux only (16 ng/ml). Expts A and B are experiments carried out on separate occasions. The distortion from linearity in ○–○ is probably due to the fact that little of the initial radioactivity is left in the cells and that the medium level of glycine is near that in the cells.

following three reasons. (1) The data show (Fig. 3) that there is only a single slope in the graphical analysis of the efflux in contrast with the experimental results reported by Christensen and Handlogten [31]. The value of the first order rate constant, k, for efflux varied between 0.04 and 0.07 min^{-1} in well over 20 experiments with control preparations of cells. In rotenone treated cells k was greater than 0.13. (2) If efflux is measured in an isotonic choline chloride medium rather than in Krebs–Ringer, there is no increase in the rate constant for efflux. Most of the recapture would be eliminated in the latter condition. (3) If efflux is carried out in a volume 1000 times the cellular volume (to minimize recapture), identical results are obtained. (Data not shown.)

Thus with low ATP levels, an increased exodus of cellular amino acid may contribute to the reduction of the steady-state level, the time required to achieve a steady state and a lack of proportionality between the initial rate of uptake and

the steady-state level. Heinz [32] has already shown that in freshly prepared Ehrlich ascites cells the steady-state position for glycine is proportional to the initial rate of transport.

The transmembrane potential and glycine uptake

Although earlier studies by Kipnis [33] appeared to eliminate the possibility that a transmembrane potential was an important factor in neutral amino acid uptake in mammalian tissues, recent reports on the action of valinomycin on amino acid uptake in Ehrlich ascites cells seem to be best interpreted on the basis that a membrane potential maintained by the cation pumping mechanism may be an important factor in energizing glycine accumulation [17]. In addition these studies imply that the transmembrane potential in Ehrlich cells is considerably larger than expected from direct measurements [34] and from measurements of the Cl^- distribution [10, 35, 36]. Since valinomycin [37, 38] will bring the distribution of K^+ to its equilibrium position, it is possible to compute the value of the transmembrane potential from the K^+ distributions in its presence [18]. Such a value will be a maximal value under the experimental conditions, the activity of internal K^+ being assumed to be equal to its concentration.

Valinomycin causes a rapid loss of cellular ATP which can be partly restored by the addition of glucose (Fig. 4). Using valinomycin, with and without glucose,

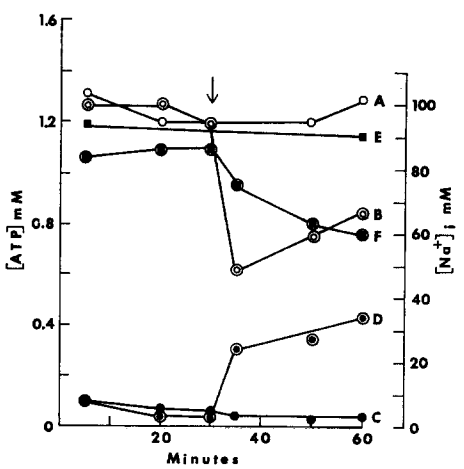

Fig. 4. Action of valinomycin on cellular Na^+ and ATP levels. Cells were preincubated for 5 min with valinomycin before samples were taken for ion and ATP determinations. The medium was 100 mM Na^+ + 65 mM K^+. The experimental procedure is given in Table II. Glucose was added at 30 min, indicated by an arrow, to a duplicate set of controls and valinomycin-containing cells. Data presented are from a typical experiment.

	ATP levels		Na_i^+ concn	
	−Glucose	+Glucose	−Glucose	+Glucose
Control	○-○ (A)	⊙-⊙ (B)	—	—
+Valinomycin	●-● (C)	⊙-⊙ (D)	■-■ (E)	(■)-(■) (F)

TABLE II

THE ELECTROCHEMICAL POTENTIAL FOR GLYCINE AT STEADY STATE VERSUS THE ELECTROCHEMICAL POTENTIALS FOR Na^+ AND K^+: ACTIONS OF VALINOMYCIN AND GLUCOSE

Cells were incubated with 0.1 mM [^{14}C]glycine (Expt I–IV) or 0.1 mM 2-aminoisobutyrate (Expt V). Incubations were for 60 min at 37 °C. Flasks B and C contained 5 μg/ml valinomycin, which was added 5–10 min before the addition of the ^{14}C-labelled amino acid. Samples were taken at 5, 20, and 30 min after the addition of glucose. At 30 min, glucose, sufficient to bring the medium concentration to 15 mM, was added to C, and samples taken at 35, 50, and 60 min. The incubation medium was 100 mM Na^+ and 65 mM K^+ with HEPES buffer (pH 7.4). The values given are the 30-min values for A and B, and 60-min values for C (30 min after addition of glucose). All ions were at steady state. Glycine uptake in C was still increasing whereas in A and B, the values were at steady state.

Expt No.		Valinomycin	Chemical potential for glycine (j/mole)	Na^+ electrochemical potential (j/mole)	Na^+ chemical potential (j/mole)	K^+ chemical potential (j/mole)	Measured cellular Na^+ concn (mM)	% decrease in cellular Na^+ required to balance energy requirements for amino acid uptake
I	A	−	6152		1841	1456	63	
	B	+	2583	996	127	869	95	45
	C	+	4462	2976	1078	1898	65	43
II	A	−	6152		2300	2748	40	
	B	+	4101	1930	434	1496	84	60
	C	+	5926	3109	792	2317	73	68
III	A	−	6398		1743	2633	50	
	B	+	2050	1415	299	1116	115	43
	C	+	4560	3063	898	2165	71	46
IV	A	−	6972		2571	2723	36	
	B	+	2870	1685	209	1476	92	43
	C	+	4786	3440	967	2473	68	44
V	A	−	7271		2994	2666	29	
	B	+	3728	2161	381	1780	86	47
	C	+	5914	3719	1328	2391	59	58

one can estimate whether sufficient energy is available from the electrochemical potential of Na^+ to account for glycine accumulation. Since the K^+ distribution is at equilibrium with valinomycin, no contribution from the potassium gradient needs to be considered.

The results in Table II show that the electrochemical potential of Na^+ is inadequate to account for glycine and 2-aminoisobutyrate accumulation if it is assumed that (a) the stoichiometry between the Na^+ and amino acid uptake is 1 : 1; (b) that the activity of the cytosol Na^+ is equal to the measured Na^+ concentration and (c) that Na^+ and the amino acid are uniformly distributed in the cellular water. The data in Fig. 4 show the changes in cellular Na^+ and ATP that occur with valinomycin and glucose.

DISCUSSION

Recent work on the mode of energizing non-electrolyte transport has led to conflicting conclusions more often than conflicting experimental observations. In essence the central question is the directness of the role of ATP or some equivalent compound in solute transport. The advocates of the indirect role point to the fact that (1) movement of an organic solute can occur against its own concentration gradient in absence of ATP provided that cation gradients are available, (2) that the system for transport is reversible and that the pumping into and out of the cells depends on the direction of the monovalent cation gradients [1–8]. Similarly, it has been concluded that inhibitors of ATP formation are without effect on initial rates, prior to a dissipation of the ion gradients [4]. In a recent communication Goldner et al. [29] showed that the initial rate of uptake in an intestinal mucosa preparation was nearly the same with and without ATP provided near normal gradients were present, whereas the initial transmural net flux was very sensitive to metabolic inhibitors.

In this communication we show that the initial rate of glycine transport is responsive to the cellular level of ATP even when ion gradients of similar magnitude are compared. In tumour cells, there appears to be agreement that at steady state higher accumulation of amino acids is obtained at higher ATP levels [10, 18, 21]. Baker and Potashner [39] have also reported that in squid axon ATP increases glutamate influx at constant ion gradients. Gibb and Eddy [17] recently suggested that the difference in amino acid accumulation between ATP-containing and ATP depleted cells at similar ion gradients is due to the size of the transmembrane potential. Such a conclusion appears unlikely for the following reasons.

(1) Elevation of extracellular K^+ to 65 mM has only a modest effect on glycine accumulation in these cells [21].

(2) The K^+ distribution is likely to be very close to equilibrium [34] at 65 mM $K_0{}^+$, and therefore the membrane potential may be computed from the K^+ distribution. Recomputation of the data in Table I, Expt. II, shows that at low ATP levels, the membrane potential would be only 10 mV less negative than at high ATP levels. A change in potential of this magnitude would be inadequate to account for the 75 % (or more) decrease in accumulation that is observed in cells with low ATP [9].

The experiments with valinomycin show that the electrochemical potential for Na^+ is an inadequate energy source for glycine and 2-aminoisobutyrate accumulation, unless very serious discrepencies exist in the estimation of the cytosol Na^+ concentra-

tion. There is reason to believe that the true cytosol Na^+ may be considerably less than the measured concentration. According to Pietrzyk and Heinz [40] a sizeable fraction of the cell Na^+ is combined with nuclear material. Thus, our conclusion concerning the available energy is tentative and awaits better estimations of the distribution of intracellular Na^+. However, it is evident from Table II (Expts B and C) that a reduction of cellular Na^+ by as much as 50 % would only bring the system into thermodynamic equilibrium even assuming 100 % coupling and efficiency of energy coupling. Although the prior estimates [22] of the degree of coupling between Na^+ and amino acids have been revised upwards (See paper by Heinz and Geck [42]), the efficiency is still less than 100 %. If the principles proposed by Kedem and Caplan [41] for energy transfer in coupled flows apply, a reduction in coupling from 100 to 90 % would result in a decrease in maximum energy transfer of nearly 60 %.

At present our data is more consistent with a direct utilization of ATP to energize amino acid transport. The following scheme is proposed based on the present and earlier observations [9, 21].

(1) Both Na^+ and ATP decrease the K_m of the transport mechanism for the amino acid.

(2) The transport mechanism probably spans the membrane and reaction with ATP internally changes the affinity for the amino acid at the exterior.

(3) Combination of the "activated" carrier with amino acid or with amino acid and Na^+, causes a change in the orientation of the transport mechanism so that (a) the amino acid and Na^+ face the cell interior and (b) the activated site is exposed to a phosphatase.

(4) Hydrolysis of the activated form results in a release of solutes into the cell and the "deactivated" carrier reorients to its original state.

(5) The energy for accumulation is derived from the energy of hydrolysis of ATP and the "activated" carrier.

(6) Transport of amino acids is not completely eliminated in the absence of ATP provided asymmetric distributions of Na^+ and K^+ are maintained. Accumulation against a chemical gradient will occur provided there is adequate free energy in the Na^+ electrochemical potential.

(7) Reversed transport is more readily observed in ATP-depleted than in ATP- containing cells because combination with ATP increases the asymmetry of operation of the transport system.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the Quebec Department of Education. The cheerful and careful assistance of Mlle. France Laviolette is gratefully acknowledged. To my colleagues, Drs R. Blostein, Department of Hematology, R. Poole, Department of Biology, and Mr Marco Colombini, Department of Biochemistry, my deepest appreciation for the innumerable discussions and criticism.

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