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CATION GRADIENTS, ATP AND AMINO ACID ACCUMULATION IN EHRLICH ASCITES CELLS

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SUMMARY

1. High levels of cellular Na^+ do not interfere with glycine or methionine accumulation in Ehrlich ascites cells.

2. Cellular ATP and extracellular Na^+, rather than a Na^+ gradient, are required for optimal amino acid accumulation.

3. Both extracellular Na^+ and ATP decrease the apparent K_m values for amino acid influx.

4. ATP stimulates the rate of downhill amino acid uptake.

5. In the absence of cellular ATP, fluxes induced by ion gradients only are insufficient to account for amino acid accumulation under physiological conditions.

INTRODUCTION

The prevalent school of thought on the role of cations in the active transport and accumulation of organic compounds[1-5] can be summarized in the following way:

I. Specific carriers for organic compounds exist at the plasma membrane.

II. Na^+ decreases the K_m or increases the v_{max} (or both) of the transport system for some organic compounds.

III. Na^+ and the organic compound are moved across the membrane by the carrier in some stoichiometric relationship.

IV. A Na^+–K^+-pump maintains low cellular Na^+. This system uses ATP directly.

V. The fact that cellular [Na^+] is low, and effectively replaced by K^+, facilitates dissociation and prevents the organic compound from recombining with the carrier at the cell interior. The process of uptake becomes essentially unidirectional into the cell.

VI. ATP is not required for the operation of the organic transport system as such, the energy for uptake of the organic compound being derived from the Na^+ (or Na^+ *plus* K^+) gradient(s) which are maintained by the operation of the ATP-dependent Na^+ pump.

There are several corollaries to a proposal of this type. Firstly, in addition to extracellular Na^+, a Na^+ gradient must be present for the accumulation of an organic compound against its concentration gradient. Secondly, so long as a Na^+ gradient of a magnitude comparable to that in a normal cell is present, net transport of the organic compound should be independent of cellular ATP. Thirdly, in a Na^+-free medium, and therefore in the absence of an "inward" Na^+ gradient, any residual net uptake of the

organic compound should be unaffected by the level of cellular ATP. Fourthly, when the external [Na^+] and the Na^+ gradient are maintained constant, the rate of equilibration of an organic compound across the cell membrane should be independent of cellular ATP.

We have recently carried out experiments with Ehrlich ascites cells to test these corollaries and have found the results to be inconsistent with some of the current views on cations and the transport of organic compounds. Our data suggest that ATP is directly required for net amino acid transport and not as a consequence of operating the Na^+ pump to maintain low cellular Na^+. Some of our earlier observations which were concerned with this subject[6] are supported and extended in the present work.

MATERIALS AND METHODS

Ehrlich ascites cells, maintained and transplanted in Swiss white mice, were isolated and prepared for experiments as described previously[7]. In this series, all cells were preincubated for 60 min prior to being used to measure transport. During this 60-min period, the cellular cation composition and/or ATP was varied.

In all experiments wet and dry weights of the cell samples were determined and corrections were made for extracellular water (inulin space). The extracellular space, which did not vary with the experimental conditions, was found to be 2.0 $\pm$ 0.2 (S.D.) μl/mg dry weight. The cell water did vary with the experimental conditions and ranged from 3.7 $\pm$ 0.2 (S.D.) μl/mg dry weight for unswollen (fresh) cells to 7.0 $\pm$ 0.3 (S.D.) μl/mg dry weight for cells maintained at 10° in media in which K^+ replaced all the Na^+.

To obtain cells with high cellular Na^+ and normal ATP levels, the preincubations were conducted in normal Ringer medium at 10°. Preincubation at 37° in a medium in which K^+ replaced all the Na^+ sustained normal ATP levels but elevated cellular K^+ and reduced cellular Na^+.

To reduce the ATP level, cells were preincubated for 60 min at 37° with $1 \cdot 10^{-4}$ M 2,4-dinitrophenol in normal Krebs–Ringer or in media in which K^+ replaced all the Na^+. This resulted in high cellular Na^+ and K^+, respectively.

To elevate the ATP levels after treatment with 2,4-dinitrophenol, the cells were transferred to fresh medium in the absence of 2,4-dinitrophenol. The addition of 10 mM glucose resulted in a rapid restoration of ATP to near normal levels well within 2–5 min of incubation[6].

To measure the movement of amino acids against their respective concentration gradients in ATP-depleted cells, we adopted the following procedure. Cells were preincubated with 2,4-dinitrophenol to deplete them of ATP. Then the radioactive compound was added and samples taken periodically until a steady state level was reached. After the latter was achieved, the intracellular concentration of radioactive amino acid was estimated (corrections being applied for extracellular space *etc.*). The cells were then transferred to fresh medium of known ionic composition with or without 2,4-dinitrophenol or glucose, as indicated in the tables, and the radioactive compound was added at the same concentration and specific activity as that found intracellularly. In this way any change in cellular radioactivity unassociated with an equivalent change in water content indicated movement against a chemical gradient.

We used [^{14}C]thiourea as a marker for nonspecific fluxes. Thiourea appears to

distribute itself readily in the cell water, the intracellular concentration reaching that of the medium. Metabolic conditions did not affect this distribution of thiourea.

The buffer used in all these experiments was 10 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonate (Hepes), pH 7.4. The "normal" incubation medium (normal Krebs–Ringer) contained 145 mM NaCl, 5.8 mM KCl, 1.5 mM KH_2PO_4 and 1.5 mM $MgSO_4$.

ATP was estimated by a coupled enzyme assay[8,9]. Na^+ and K^+ were estimated by flame photometry. The measurement of radioactivity and sampling technique were described previously[7]. Labelled compounds were obtained from New England Nuclear Corp., Waltham, Mass. The labelled compounds used were L-[Me-^{14}C]methionine; [1-^{14}C]glycine; L-[1-^{14}C]leucine and [^{14}C]thiourea.

RESULTS

Transport in cells containing ATP

The contribution of the Na^+ gradient to the transport of organic compounds may be examined either by reducing extracellular Na^+ or increasing cellular Na^+. Since extracellular Na^+ is known to be required for the active transport of many organic compounds[1–3,10–16] we chose to examine the effect of the Na^+ gradient by elevating the cellular Na^+ concentration. Two possible means of elevating cell Na^+ are: (a) lowering the temperature of incubation or (b) adding metabolic inhibitors to abolish ATP production. The former treatment alters ATP concentration relatively little, while the latter, of course, greatly reduces the ATP level. The ATP level can be rapidly restored to near normal values by transferring the poisoned cells to fresh, inhibitor-free medium containing glucose. ATP is elevated[6] long before the normal ion gradients are reestablished (Table I).

Using the preincubation conditions outlined in Table I, experimental conditions were obtained which permitted us to ascertain whether accumulation of methionine is associated primarily with the magnitude of the cation gradients or the level of cellular ATP. K^+ was used as the only Na^+ replacement ion in this series. It has been shown[17–19] that the sum of the Na^+ *plus* K^+ concentrations in these cells remains, despite swelling, about 200 $\pm$ 10 mM, a figure which we also confirmed. Therefore, the cellular K^+ content can be readily estimated and the K^+ gradient computed.

The results in Table I show that net uptake* of methionine is much more affected by changes in the cellular ATP levels than by changes in the magnitude of the Na^+ (or $Na^+ + K^+$) gradient(s). Comparable accumulation of methionine is obtained with and without a Na^+ gradient provided that ATP and extracellular Na^+ are present. In cells with cation gradients of equal magnitude but with different ATP concentrations, accumulation decreases with decreasing concentrations of ATP. Similar data are obtained with glycine (Table II). Moreover, the level of cellular Na^+ has little effect on net methionine uptake in the presence of either normal or low extracellular levels of Na^+ (Table III).

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* In preincubated cells, the uptake due to exchange diffusion would not exceed 6 nmoles/mg dry wt. based on data obtained from analysis of free amino acid pool using a Technicon amino acid analyzer.

TABLE I

THE Na^+ GRADIENT, ATP AND THE UPTAKE OF METHIONINE FROM NORMAL KREBS–RINGER MEDIUM

Cells were preincubated in media shown for 60 min and subsequently transferred to normal Krebs–Ringer medium containing 145 mequiv/l Na^+ and 8 mequiv/L K^+, 2 mM L-[Me-^{14}C]methionine: specific activity 125 counts/min per nmole. Hepes buffer, pH 7.4, was used. Incubation was for 30 min at 25°. Samples were taken at intervals between 0 and 30 min. Data for the 30-min values are given. 2,4-Dinitrophenol, where used, was at $1 \cdot 10^{-4}$ M. To elevate the ATP levels after treatment with 2,4-dinitrophenol, 10 mM glucose was added. The data given are typical of that obtained in three separate experiments. In all experiments wet and dry weight measurements were performed and cell volume determined. t_0 and t_{30} represent the readings at 0 time and 30 min respectively.

Preincubation conditions	*Medium Na^+ (μequiv/ml)*	*Cellular Na^+ (μequiv/ml cell water)*		*Cellular ATP (μmoles/ml cell water)*		*L-[Me-^{14}C]methionine (uptake* (nmoles/mg dry wt.)*
		t_0	t_{30}	t_0	t_{30}	t_{30}
37° Krebs–Ringer	145	20–30	35–50	2.1	2.8	24.0
10° K^+–Ringer	145	2–4	35–50	2.1	2.8	26.0
10° Krebs–Ringer	145	170–180	140–160	2.3	3.1	25.0
37° Krebs–Ringer + 2,4-dinitrophenol	145	155	110	1.6**	1.9**	25.0
37° Krebs–Ringer + 2,4-dinitrophenol	145	155	145	0.3	0.4	12.0
37° Krebs–Ringer + 2,4-dinitrophenol	145	155	150	$\leqslant$0.1***	$\leqslant$0.1***	8.2

* In fresh cells, the cell water was estimated to be 3.65 $\pm$ 0.04 (S.D.) μl/mg dry wt. Therefore in unswollen cells a cellular concentration of 2 mM is equivalent to 7.4 nmoles/mg dry wt.

** The incubation medium contained 10 mM glucose.

*** The incubation medium contained $1 \cdot 10^{-4}$ M 2,4-dinitrophenol.

TABLE II

THE Na^+ GRADIENT, ATP AND THE UPTAKE OF GLYCINE FROM NORMAL KREBS–RINGER MEDIUM

The experimental conditions are as outlined in Table I. 2 mM [1-^{14}C]glycine, specific activity 125 counts/min per nmole was used.

Preincubation conditions	*Medium Na^+ (μequiv/ml)*	*Cellular Na^+ (μequiv/ml cell water)*		*Cellular ATP (μmoles/ml cell water)*		*[1-^{14}C]glycine uptake* (nmoles/mg dry wt.)*
		t_0	t_{30}	t_0	t_{30}	t_{60}
37° Krebs–Ringer	145	20–30	—	3.5**	3.1**	82.1
37° Krebs–Ringer + 2,4-dinitrophenol	145	150–160	—	2.5**	2.7**	74.7
37° Krebs–Ringer + 2,4-dinitrophenol	145	150–160	—	0.2	0.4	23.0
37° Krebs–Ringer + 2,4-dinitrophenol	145	150–160	150–160	$\leqslant$0.1***	$\leqslant$0.1***	16.4

* A cellular concentration of 2 μmoles/ml cell water of glycine is equivalent to 7.4 nmoles/mg dry wt. in the unswollen cell.
** The incubation medium contans 10 mM glucose.
*** The incubation medium contans $1 \cdot 10^{-4}$ M 2,4-dinitrophenol.

TABLE III

THE EFFECT OF EXTRACELLULAR Na^+ AND THE Na^+ GRADIENT ON METHIONINE UPTAKE

Conditions as described in Table I. In this experiment, choline chloride replaced the Na^+. K^+ was maintained at 8 mM. Where indicated $1 \cdot 10^{-4}$ M 2,4-dinitrophenol was present in the preincubation and incubation periods.

Preincubation conditions	*Medium Na^+ (μequiv/ml)*	*Cellular Na^+ (μequiv/ml cell water)*		*Cellular ATP (μmoles/ml cell water)*		*L-[Me-^{14}C]methionine uptake* (nmoles/mg dry wt.)*
		t_0	t_{60}	t_0	t_{60}	t_{60}
37° Krebs–Ringer	145	20–30	35–50	2.5	3.1	43.0
37° Krebs–Ringer	0–5	20–30	10–20	2.4	2.8	10.1
37° Krebs–Ringer + 2,4-dinitrophenol**	145	150–160	80–100	1.7	2.0	35.7
37° Krebs–Ringer + 2,4-dinitrophenol**	0–5	150–160	10–20	1.6	1.9	8.7

* A cellular concentration of 2 μmoles/ml cell water of methionine is equivalent to 7.4 μmoles/mg dry wt. in the unswollen cell.
** The medium contained 10 mM glucose.

TABLE IV

THE Na^+ *plus* K^+ GRADIENT-INDUCED FLUX OF METHIONINE, GLYCINE, LEUCINE AND THIOUREA IN ATP-DEPLETED CELLS

Cells were depleted of ATP by incubation in $1 \cdot 10^{-4}$ M 2,4-dinitrophenol at 37° in media of specified ionic composition (see MATERIALS AND METHODS). The radioactive compounds were added after 30 min and the preincubation continued until the intracellular radioactivity approached the steady state (about 30–40 min). The cells were then centrifuged and added to fresh incubation medium which contained $1 \cdot 10^{-4}$ M 2,4-dinitrophenol in addition to the ionic compositions given in the Table. [1-^{14}C]Glycine, L-[1-^{14}C]leucine, [^{14}C]thiourea and L-[Me-^{14}C]methionine were added to the incubation medium at the same concentration (3 mM) and specific activity (120 counts/min per nmole) as used during the preincubation period.

Extracellular (*μequiv/ml*)		*Cellular* Na^+ (*μequiv/ml cell water*)		*Cellular* K^+ (*μequiv/ml cell water*)		*Net uptake (2–30 min)** (*nmoles/mg dry wt.*)			
Na^+	K^+	t_2	t_{30}	t_2	t_{30}	*Methionine*	*Glycine*	*Leucine*	*Thiourea*
145	8	30–60	90–135	105–165	30–85	5.0	8.8	2.9	2.5
145	8	170–210	160–200	10–15	10–15	1.6	3.2	—	—
Nil	153	10–25	5–15	165–185	150–165	−2.1	−0.8	—	—
Nil	153	90–135	50–110	80–95	85–120	−6.9	−9.8	−4.1	−0.7

* *Minus* signs denote efflux.

Cation gradients and amino acid transport in cells depleted of ATP

In ATP-depleted cells, it is possible to obtain ion gradients by judicious adjustment of the intra- and extracellular ion concentrations (see MATERIALS AND METHODS). It has already been shown that net amino acid movement against a concentration gradient occurs under the influence of cation gradients in absence of cellular ATP[20–22]. The question that has not been satisfactorily answered is whether the rate of amino acid uptake under the influence of cation gradients (in ATP-depleted cells) is sufficient to account for the accumulation of amino acids under more physiological conditions.

To examine this question we had to overcome certain technical difficulties. In freshly isolated Ehrlich ascites cells, exchange diffusion with endogenous amino acids complicates the assessment of net amino acid, particularly methionine uptake[6]. At low temperatures (25° or less) and with low levels of extracellular sodium, cation-insensitive exchange, though small[6,23–25], may be significant relative to the total uptake. If the external amino acid concentration is low, uptake by exchange may appear to be accumulative and against a gradient. For this reason and to increase the sensitivity of our assay, ATP-depleted cells were preequilibrated with ^{14}C-labelled amino acids or [^{14}C]thiourea prior to measuring net fluxes due to cationic gradients (see MATERIALS AND METHODS). In our experimental design any change in ^{14}C-labelled amino acid concentration reflects net movement against a concentration gradient. Thiourea was used as an inert marker to estimate nonspecific fluxes[26].

The results in Table IV show that in ATP-depleted cells a modest change in the concentration of cellular amino acids occurs in the presence of a Na^+ *plus* K^+ gradient. Methionine, and glycine fluxes occur against their respective gradients but along the Na^+ gradient and against the K^+ gradient. (Compare lines 1 and 2; 3 and 4 in Table IV.) Leucine flux is small compared with that of methionine or glycine. Individual cations gradients are less effective than the combined ion gradients in causing net flux of methionine against its concentration gradient (Table V). (Compare the differences

TABLE V

Na^+ GRADIENT AND K^+-GRADIENT INDUCED FLUX OF METHIONINE AND THIOUREA IN ATP-DEPLETED CELLS

*Cation***	*Extracellular concentration (μequiv/ml)*	*Initial cellular concentration (μequiv/ml cell water)*	*Cation gradient*			*Net uptake (Δ 2–30 min)** *(nmoles/mg dry wt.)*	
			Direction	*Magnitude (μequiv/ml)*		L-[*Me*-^{14}*C*]*methionine*	[^{14}C]*thiourea*
				t_2	t_{30}		
Na^+	145	20–30	inward	80–120	10–60	0.6	−1.4
	145	160–180	outward	10–40	0–30	−0.6	−0.5
	Nil	20–30	outward	10–30	10–20	−2.0	1.3
	Nil	160–180	outward	140–180	50–110	−3.6	0.6
K^+	8	165–175	outward	100–150	30–80	−0.1	1.1
	153	165–175	outward	15–25	0–10	−1.7	—
	8	5–25	outward	0–15	0–10	−2.4	—
	153	5–25	inward	60–100	40–70	−4.1	−1.4

* A *minus* sign denotes efflux.
** Choline chloride was used as the replacement salt so that only Na^+ or K^+ was present at any one time.

TABLE VI

THE EFFECT OF ATP ON THE UPTAKE OF GLYCINE, METHIONINE, LEUCINE AND THIOUREA INTO CELLS WITH NORMAL Na^+ *plus* K^+ GRADIENTS

ATP-depleted cells, containing known concentrations of the radioactive compounds and high K^+ were obtained as described in MATERIALS AND METHODS and Table IV. These cells were transferred to fresh, normal Krebs–Ringer medium containing $1 \cdot 10^{-4}$ M 2,4-dinitrophenol and the radioactive amino acid at the same concentration and specific activity as that found intracellularly at the end of the preincubation period. Generally the concentration and specific activities were 3 mM and 120 counts/min per nmole, respectively. The incubations were carried out at 25° and samples were taken at several intervals over a 30-min period. Cells with normal ATP levels were used for comparative purposes. The latter cells were preincubated in normal Krebs–Ringer for the same period of time as the ATP-depleted cells. The cells were transferred to fresh medium and incubated with the same concentration and specific activity of amino acids as that used with ATP-depleted cells. Samples were taken until the intracellular concentration of radioisotope reached that of the medium. This point was considered t_0 and equivalent to the starting point with the ATP-depleted cells. Incubation was at 25° with samples taken at intervals over a 30-min period.

Additions	*Inward Na^+ gradient* (*μequiv/ml*)	*Outward K^+ gradient* (*μequiv/ml*)	*Extracellular 2,4-dinitrophenol* (*μmoles/ml*)	*Net uptake between 2 and 5 min* (*nmoles/mg dry wt.*)
[1-^{14}C]Glycine	95–105	145–155	Nil	8.4
	70–100	85–145	0.1	0.8
L-[Me-^{14}C]Methionine	95–105	145–155	Nil	3.7
	70–100	85–145	0.1	0.5
L-[1-^{14}C]Leucine	95–105	145–155	Nil	1.4
	70–100	85–145	0.1	0.3
[^{14}C]Thiourea	95–105	145–155	Nil	0.3
	70–100	85–145	0.1	0.5

between lines 1 and 2; 3 and 4; 5 and 6; and 7 and 8 in Table V with the differences between lines 1 and 2 and 3 and 4 in Table IV.) The data in Table VI show that in the presence of Na^+ *plus* K^+ gradients of similar magnitude, the uptakes of methionine and glycine are considerably smaller in ATP-depleted cells than in cells containing ATP. Nonetheless, the data support the conclusion that the Na^+ *plus* K^+ gradients will drive amino acid fluxes in either direction in ATP-depleted cells, albeit to a limited extent.

In our hands, however, efflux of an amino acid from the cell along a Na^+ gradient did not occur unless the reversed Na^+ gradient was in excess of 100 mM (Fig. 1). Thus when ATP-depleted cells containing 150–180 mM Na^+ were placed in media containing 80 mM Na^+, no efflux against a gradient occurred. However, if the cells were placed in media which contained only 20 mM Na^+, then efflux was significant. The requirement for a large reversed Na^+ gradient to drive amino acid efflux is even more apparent in cells containing ATP. Thus efflux is observed in media containing 20 mM Na^+, but appreciable net accumulation against the Na^+ gradient as well as against the amino acid gradient is observed in media containing 80 mM Na^+ (Fig. 1). Uptake of amino acids against the Na^+ gradient has been reported by others[27–29]. The data in Fig. 1 permit a comparison between the "ion gradient induced transport" and the "ATP *plus* ion gradient induced transport" of amino acids. The contribution of ATP is striking.

Net uptake of methionine along its concentration gradient

The Na^+ gradient hypothesis provides no role for ATP in the equilibration of organic substances between cell and medium. Thus rate of amino acid uptake before equilibration and the time required to attain equilibration should be independent of

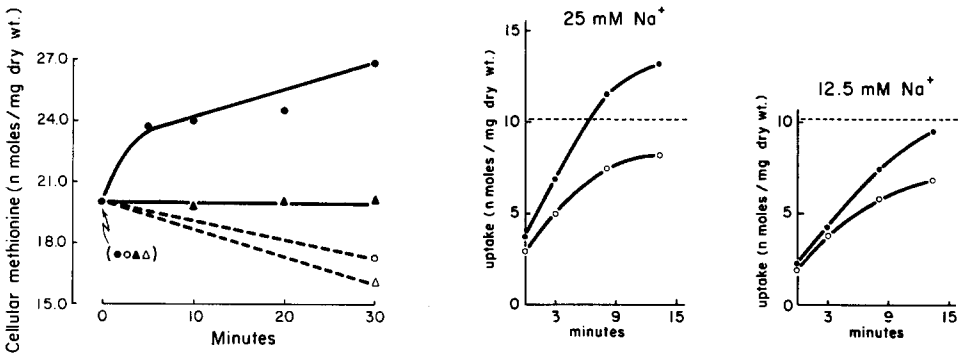

Fig. 1. The cells were preincubated for 1 h at 10° in normal Krebs–Ringer or at 37° in Krebs–Ringer containing $1 \cdot 10^{-4}$ M 2,4-dinitrophenol. The medium contained 3 mM L-[Me-^{14}C]methionine, specific activity 120 counts/min per nmole. This gave rise to cells which contained nearly equal Na^+ and methionine levels but greatly different ATP levels. One lot of cells from each group was transferred to media containing 20 mM Na^+ or 80 mM Na^+ and the same concentration and specific activity of methionine as that found in the cells after preincubation. Na^+ was replaced by choline. Changes in [^{14}C]methionine concentration were measured over a period of 30 min at 25°. ●—●, ATP and 80 mM Na^+; ▲—▲, 2,4-dinitrophenol and 80 mM Na^+; ○—○, ATP and 20 mM Na^+; △—△, 2,4-dinitrophenol and 20 mM Na^+.

Fig. 2. Cells were preincubated at 37° for 1 h in modified Ringer solutions containing $1 \cdot 10^{-4}$ M 2,4-dinitrophenol in which K^+ replaced Na^+. This gave rise to cells with near normal K^+ levels and little ATP. After preincubation, the cells were divided into two lots. To one, were added $1 \cdot 10^{-4}$ M 2,4-dinitrophenol and 10 mM pyruvate. To the other were added 1 mM glucose, 10 mM pyruvate but no 2,4-dinitrophenol. The media contained the Na^+ concentrations shown at the top of the figure and isotonicity was maintained with choline chloride; K^+ was kept at 8 mM. The cells were incubated for 2 min at 25° and then 2.5 mM L-[Me-^{14}C]methionine, specific activity 180 counts/min per nmole, was added and uptake of radioactivity measured. ●—●, glucose, no 2,4-dinitrophenol; ○—○, 2,4-dinitrophenol. The dashed lines indicate the position where the intracellular and extracellular concentrations become equal.

the ATP concentration in cells with a Na^+ gradient of given magnitude. To overcome the contributions from exchange diffusion with endogenous amino acids which would distort the true value for net amino acid uptake, we depleted the cells of endogenous amino acids. We found that preincubation for 1 h at 37° in Na^+-free media containing 2,4-dinitrophenol leads to a major reduction in the free amino acid pool from 100 nmoles/mg dry wt. to 5.5 nmoles/mg dry wt. Such cells can recover their ability to accumulate amino acids but exchange with endogenous amino acids is greatly reduced and it is possible to measure net methionine (and glycine) uptake as function of time before a 1:1 distribution is reached. In Figs. 2 and 3 are shown the results obtained. It takes longer to achieve equilibration of amino acids in ATP-depleted cells than in cells containing ATP. ATP increases the rate of amino acid uptake along the concentration gradient. A similar result was reported by MANNO AND SCHACHTER[30] for ATP-dependent galactoside transport in *Escherichia coli* preparations.

In Na^+-free media, cells depleted of endogenous amino acids and ATP do not take up methionine at a rate sufficient to equilibrate the amino acid between cell and

medium in 2 h of incubation. This slow rate of uptake is still largely carrier mediated since it may be depressed by ethionine. If the ATP level is elevated (by the addition of glucose), equilibration is attained within 2 h in Na^+-free media but net accumulation against a concentration gradient is rarely observed (Table VII).

TABLE VII

THE UPTAKE OF METHIONINE INTO CELLS DEPLETED OF ATP AND ENDOGENOUS FREE AMINO ACIDS

The cells were preincubated at 37° for 60 min in a modified Krebs–Ringer solution in which Na^+ was replaced by K^+ and which contained $1 \cdot 10^{-4}$ M 2,4-dinitrophenol. The cells were then transferred to Na^+-free media containing $1 \cdot 10^{-4}$ M 2,4-dinitrophenol in which choline replaced Na^+. 2 mM methionine of specific activity given in Table I. Incubation at 25°. Other conditons as in Table I.

Extracellular L-[*Me*-^{14}C]*methionine* (*μmoles/ml*)	*Cellular ATP* (*μmoles/ml cell water*)	*Uptake of* L-[*Me*-^{14}C]*methionine* (*μmoles/ml cell water per 2 h*)
2.0	$\leqslant$ 0.1*	0.4
2.0 + 20 mM L-ethionine	$\leqslant$ 0.1*	0.2
20.0	$\leqslant$ 0.1	3.8
2.0	> 1.6**	2.1

* Incubation medium contained $1 \cdot 10^{-4}$ M 2,4-dinitrophenol.
** Incubation medium contained 10 mM glucose and no 2,4-dinitrophenol.

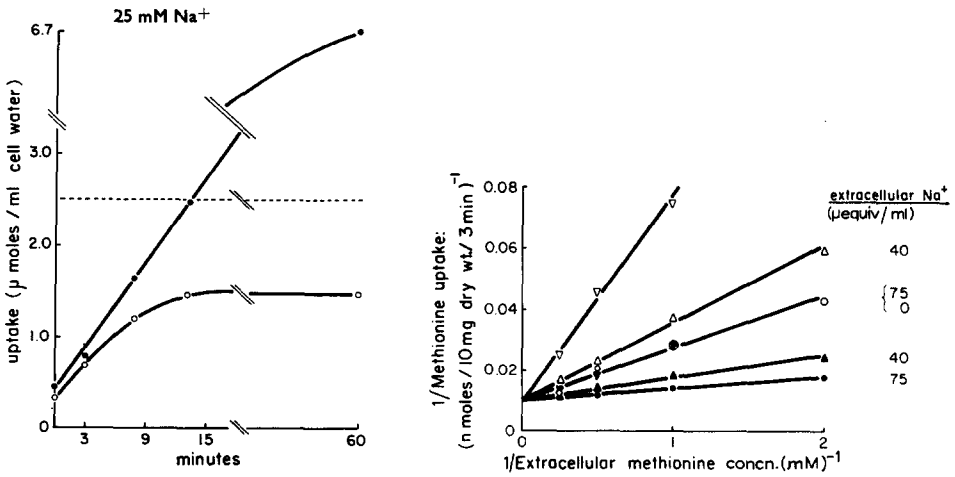

Fig. 3. Conditions were identical to those described for Fig. 2 except that 2.7 mM [1-^{14}C]glycine, specific activity 60 counts/min per nmole, was used. ●—●, glucose, no 2,4-dinitrophenol; ○—○, 2,4-dinitrophenol.

Fig. 4. Cells were preincubated for 60 min at 37° in normal Krebs–Ringer (ATP-containing cells) or in modified medium with K^+ replacing Na^+ and containing $1 \cdot 10^{-4}$ M 2,4-dinitrophenol (ATP-depleted cells). All preincubations were carried out with 0.01 mM [^{14}C]methionine of the same specific activity as that to be used in the subsequent incubation. This pretreatment with amino acid replaced the "exchangaeble pool" with an amino acid of known specific activity and was used as the baseline above which the initial rate of net amino acid uptake was measured during the experimental period. After the preincubation, the cells were transferred to fresh medium containing [Na^+] as noted in the figure. Cells pretreated with 2,4-dinitrophenol were maintained in 2,4-dinitrophenol. Choline was used as the replacement ion and [K^+) was 8 mM. Uptake was measured over a period of 5 min at 25°. The initial uptake was computed from the values at 3 min since the rate of uptake was nearly constant for 4–5 minutes. Specific activity of methionine as given in Table I. Closed symbols, cells containing ATP. Open symbols, ATP-depleted cells.

Kinetic parameters

The data presented in Figs. 4 and 5 show that both Na^+ and ATP decrease the apparent K_m values for glycine and methionine uptake. The apparent maximum uptakes are relatively unaffected by Na^+ or ATP.

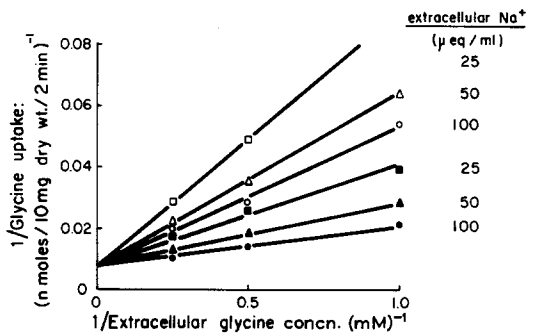

Fig. 5. Conditions as in Fig. 4 whith [1-^{14}C]glycine as the test amino acid. Closed symbols, cells with ATP; open symbols, ATP-depleted cells.

TABLE VIII

EFFECT OF ATP ON THE Na^+ REQUIREMENT FOR GLYCINE AND METHIONINE UPTAKE

Conditions as described in Figs. 4 and 5.

Cell Type	*Test amino acid*	*K_m for Na^+ (mM)*
ATP-containing	[1-^{14}C]glycine, 1 mM	31
	2 mM	31
ATP-depleted	[1-^{14}C]glycine, 1 mM	31
	2 mM	31
	3 mM	31
ATP-containing	L-[Me-^{14}C]methionine, 1 mM	13
	2 mM	13
	4 mM	13
ATP-depleted	L-[Me-^{14}C]methionine, 2 mM	13
	4 mM	13

The initial rates of amino acid uptake from Figs. 4 and 5 as well as additional data from other experiments were plotted as a function of $1/[Na^+]$ to determine the effect of ATP on the Na^+ requirement for amino acid uptake. The data in Table VIII show that (a), ATP does not alter the apparent K_m for Na^+ and (b), higher concentrations of Na^+ are required to obtain 50 % saturation with respect to Na^+ for glycine* uptake compared with that for methionine. This no doubt accounts for the previous observations that glycine uptake is much more sensitive to a reduction in external Na^+ concentration than is that of methionine[6].

* It is interesting to note that EDDY AND HOGG[39] obtained a similar K_m value for Na^+.

DISCUSSION

The hypothesis that the Na^+ gradient provides the energy for the transport of organic compounds is most attractive because of its parsimony in the expenditure of metabolic energy.

Several investigators have commented that sufficient energy is available from Na^+ (or the Na^+ *plus* K^+) gradient(s) to achieve the accumulation of organic compounds observed experimentally[2,19,21,31,32]. EDDY[19] showed that the uptake of amino acids by CN^--poisoned cells was less than that of respiring cells despite the fact that both types of cells possessed Na^+ gradients of approximately equal magnitude. EDDY[19] suggested that the K^+ gradient may be the additional driving force. JACQUEZ AND SHAFER[31] demonstrated that if the sum of the Na^+ *plus* K^+ gradients is considered as the energy source, sufficient energy is indeed available from those gradients for amino acid accumulation except when the extracellular concentration of K^+ is high (J. A. JACQUEZ, private communication of data from ref. 31, experiments Nos. 11–15, 18 and 19). When the K^+ gradient is abolished by elevating extracellular K^+, the uptake of amino acid is nearly equal to or in excess of the theoretical gradient energy available.

In the present experiments we have compared amino acid transport in cells containing low and high levels of ATP but with cation (Na^+ *plus* K^+) gradients of nearly equal magnitude as well as in cells wherein the ATP levels were constant but the ion gradients were varied over a wide range. We have found that the uptake of amino acids is dependent on the ATP level of the cell and not on the magnitude of the ion gradients. (In Table I, compare line 2 with 3, 3 with 5 and 4 with 5.) Despite the fact that in ATP-depleted cells, Na^+ plus K^+ gradients will bring about a modest flux of amino acids against their concentration gradients, the flux is small compared with that observed in cells containing ATP.

It has been suggested that fluxes of organic compounds are modest in cells deprived of ATP because the ion gradients are rapidly dissipated[22]. We therefore tested amino acid fluxes in ATP-depleted cells over short periods of time during which the dissipation of the gradient was nominal (Table VI). The data given in Table VI cite the values of the average magnitude of the gradient over the experimental period. It seems unlikely that dissipation of the energy from the gradient is the major reason for the relatively small amino acid uptake in cells depleted of ATP.

That extracellular Na^+ increases the initial rate of uptake has been shown by others[1,2,4,20,33–36] as well as in this work. The present observation that the steady-state level of amino acids is not significantly affected by the level of intracellular Na^+ argues against a major role for cell Na^+ in the regulation of the steady state position of amino acids in these cells.

Both Na^+ and ATP appear to act on the step involved in amino acid uptake into the cell. Earlier work showed that loss of cellular amino acid is independent of ATP[37]. The present data as well as those obtained earlier show that ATP stimulates the initial rate of uptake[37]. In agreement with EDDY *et al.*[20], we also find that the maximum initial rate of amino acid uptake at saturating levels of Na^+ is not affected by the cellular ATP level.

A kinetic analysis of the present experiments has shown that both Na^+ and ATP decrease the K_m value of the amino acid for its carrier. The effect of Na^+ in this system is well documented[19,20,23–25,27,28,36,38]. If E and ε represent, respectively, the carrier

before and after activation with ATP, and A represents the amino acid, our data suggest that E, ε, ENa^+ and εNa^+ are all capable of catalyzing amino acid transport and that the K_m for the amino acids decreases in the order given above as the carrier is converted into its various forms. The spatial separation of the enzymes involved in converting $E \longrightarrow \varepsilon$ at the external surface and $\varepsilon \longrightarrow E$ at the internal surface would keep the system operating in an asymmetric manner leading to accumulation. Since the apparent K_m for Na^+ is unaffected by the cellular ATP level, it may be surmised that the dissociation constant for $ENa^+ \rightleftarrows E^+ + Na^+$ is not markedly different from that of $\varepsilon Na^+ \rightleftarrows \varepsilon + Na^+$.

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