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GLYCINE ACCUMULATION IN ABSENCE OF Na^+ AND K^+ GRADIENTS IN EHRLICH ASCITES CELLS:

SHORTFALL OF THE POTENTIAL ENERGY FROM THE ION GRADIENTS FOR GLYCINE ACCUMULATION

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SUMMARY

The contribution of the potential energy from the K^+ gradient to glycine transport in Ehrlich ascites cells was examined. Cells were pretreated in K^+-free media at 0 °C to elevate cellular Na^+. In these cells with u small (< 30 mM) Na^+ gradient, the effect of elevating extracellular K^+ was examined while extracellular Na^+ was maintained at 100 mM. The results show that glycine accumulation at steady state is little affected by elevating extracellular K^+ and that the energy required for glycine accumulation may exceed by a factor of 3 the theoretical potential energy from the cation gradients. Under optimal K^+ gradient conditions, coupling efficiency would have to exceed 70% if the cation gradients provided the energy for amino acid accumulation in cells high in Na^+_i.

INTRODUCTION

Recently there has been considerable debate on the respective roles of the monovalent cation gradients and cellular ATP on organic solute accumulation. The differences of opinion that appear to exist amongst investigators center around the relative contributions of the Na^+ gradient, the K^+ gradient and cellular ATP as energy sources for organic solute transfer against a concentration gradient.

Thus, Vidaver and coworkers[1,2], Crane *et al.*[3,4], and Schultz and Curran and coworkers[5–7] have pointed out that sufficient potential energy is available from the Na^+ gradient alone to account for the transfer of amino acids and sugars across pigeon red cell and intestinal epithelium, respectively. On the other hand, in mouse ascitic tumour cells, Riggs *et al.*[8], Jacquez and Schafer[12], and Eddy and coworkers[9–11] have concluded that the Na^+ gradient alone is inadequate and that some portion of the energy must be obtained from another source. Reid and Eddy[11] have concluded that the additional source is probably the K^+ gradient and that ATP is required as a coupling factor to transfer energy from the K^+ gradient to amino acid

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate.

accumulation. Schafer and Heinz[13] have lately reported that although there is a quantitative relationship between the energy available from the Na^+ *plus* K^+ gradients (including the transmembrane potential) and amino acid accumulation, there is a deficit of about 900 cal/mole which is probably derived directly from cellular metabolism. Kimmich[14], on the other hand, concludes that the gradients are unlikely to be sources of energy for sugar and amino acid transport in chick intestinal epithelium.

In our experimental work we have obtained relatively little evidence consistent with the conclusion that ion gradients are a major source of energy for accumulation of amino acids. Thus, Ehrlich cells, in which the Na^+ gradient is abolished[15,16] but cellular ATP is available, do not show diminished amino acid accumulation. Even if only a portion of the energy were derived from the Na^+ gradient, a decreased accumulation should be obtained. (In most of our earlier work with Ehrlich cells a K^+ gradient was maintained.)

Mouse pancreas[17] incubated *in vitro* does not maintain a significant Na^+ gradient (less than 50 mM) and alteration of the K^+ gradient by elevating extracellular K^+, does not decrease glycine accumulation so long as $[Na^+]_o$ is maintained constant. Furthermore, experimental situations may be obtained with the pancreas wherein the energy required for amino acid accumulation is at least twice the potential energy available from the monovalent cation gradients[17].

One of the frequent problems in interpretation of the data from experiments in which the magnitude of the gradients is varied, is that the external concentrations of the ions are varied concomitantly. Therefore, it may become difficult to distinguish between "gradient" effects and "concentration" effects.

Our recent approach to this problem has been as follows. Our earlier work with Ehrlich cells showed that extracellular Na^+ at 100 mM was nearly optimal for amino acid accumulation but that a Na^+ gradient was not necessary. The requirement for cellular ATP was established but the question of the contribution of the K^+ gradient energy to amino acid accumulation was not determined. If transport and accumulation of amino acids are dependent on a fraction of the energy available in the K^+ gradient, then under conditions where the Na^+ gradient is small, (less than 50 mM) but the extracellular Na^+ is constant and high enough to maintain active transport, elevation of the extracellular K^+ concentration should be reflected in a decreased uptake of amino acids. These experiments were performed and the results obtained are inconsistent with the conclusion that the energy from the K^+ gradient is being used to a major extent for glycine accumulation.

MATERIALS AND METHODS

Ehrlich ascites cells were obtained as previously described[18]. The experimental procedures for Na^+ and K^+ estimations, amino acid accumulation, wet and dry weight measurements and extracellular spaces have been described in previous communications[15,16].

In this series, all experiments to measure amino acid uptake were carried out in 100 mM Na^+, isotonicity was maintained by adding sufficient choline chloride or KCl. Thus a typical incubation medium consisted of 100 mM Na^+, 10 mM K^+, 50 mM choline and 1.5 mM Mg^{2+} with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) buffer, pH 7.4. To assess the effects of high external K^+, the choline

chloride was replaced by an equivalent concentration of KCl. To obtain cells high in Na^+, the experiments were preceded by an incubation period of 60 min in the cold or at 25 °C in K^+-free medium in which all K^+ was replaced by Na^+ except for the K^+ in the HEPES buffer. Such cells had between 50 and 80 mM Na^+ intracellularly during the actual experimental period, depending largely on the temperature at which the cells were preincubated.

RESULTS

The data in Table I show a pair of typical experiments in which the accumulation of glycine is presented as well as the intra- and extracellular cation concentrations. The cells had been subjected to different preincubations to manipulate the ion distributions. It is apparent that the magnitude of the K^+ gradient existing at the time at which glycine uptake has reached near steady state conditions is not a major factor in determining glycine distribution at steady state.

In Fig. data 1 are given from an additional series in which the logarithm of the

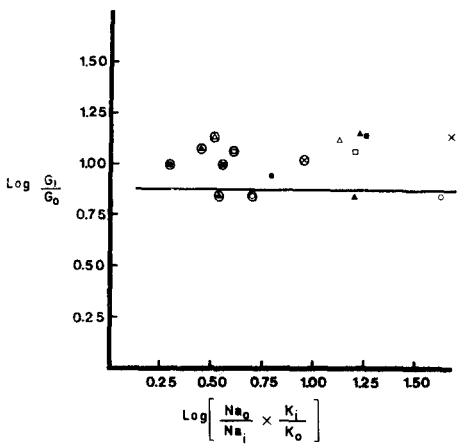

Fig. 1. Relationship at steady state between magnitude of the log of the cation gradients and the log of the glycine accumulation ratio. Each pair of experiments is indicated by a symbol and a circled symbol, *e.g.* × and ⊗, where the former represents the experiments with 10 mM K^+_o and the latter with 60 mM K^+_o. Several types of manipulations were used to vary intracellular $Na^+ + K^+$, such as preincubation at 37 °C or 0 °C in normal Krebs–Ringer, K^+-free Ringer, or isotonic NaCl. The measurements of amino acid uptake were carried out at 37 °C (values above the line) or 25 °C (values below the line). Glycine concentration throughout was 0.1 mM, specific activity 180 cpm/nmole and Na^+_o = 100 mequiv/l. The values used for calculation were those derived from the steady-state position for both ions and glycine. Samples were taken over a 90 min period to ascertain that steady state (or near steady state) had been attained.

glycine accumulation ratio at steady state was calculated and plotted against the sum of the logs of the Na^+ and K^+ gradients at steady state (a measure of the potential energy available from the ion gradients). It may be seen that a decrease in potential gradient energy of nearly 70 % does not appreciably alter the accumulation of glycine.

If the glycine transport system derives energy from the Na^+ *plus* K^+ gradients then, as the gradient energy decreases, glycine accumulation should decrease unless there is a substantial excess of energy available from the gradients. If one calculates

the potential energy available from the gradients in cells incubated in high K^+, and the energy required for glycine accumulation, it can be seen that the required energy for glycine accumulation may exceed the potential energy available by a factor greater than 3 (Table II). These data are derived from the experiments with elevated K^+ shown in Fig. 1.

Although we have reported steady state values in the data given above, the data are selected from experiments in which at least seven samples were taken over a 60–90 min period. One of the observations we made was that high extracellular K^+ appears to reduce the rate of uptake of glycine but that the steady-state level is not greatly reduced. Thus, in experiments where high K^+_o is used, an apparent effect of the K^+ gradient on amino acid accumulation may be seen unless steady state measurements of amino acid uptake are made (Fig. 2). In the report by Schafer and

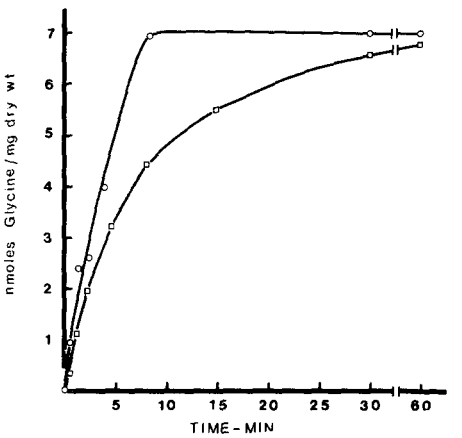

Fig. 2. Time course of glycine uptake at 37 °C in 10 and 60 mM KCl. Ehrlich ascites cells were preincubated for 60 min at 0–4 °C in K^+-free media. The cells were centrifuged, resuspended and incubated in (○) a solution containing 100 mM NaCl, 50 mM choline chloride and 10 mM K^+, or (□) 100 mM NaCl, 60 mM K^+. Incubation was carried out in HEPES buffer at pH 7.4 at 37 °C with 0.1 mM glycine, specific activity 180 cpm/nmole. The gas phase was air.

Heinz[13], an effect of the K^+ gradient on α-aminoisobutyric acid uptake was reported. The relationship between amino acid accumulation and the K^+ gradient was derived from non-steady-state conditions. Therefore, the data may indicate an inhibitory effect of K^+_o on the rate of amino acid uptake rather than a decreased availability of energy.

By assuming that the approach to steady state is a first-order process, we have calculated that the $t_{1/2}$ for the time required to achieve steady state is increased from about 3.0 to 6.0 min when K^+ is raised from 10 to 60 mM. In this regard it is interesting to note that, in cells depleted of ATP, glycine efflux from cells high in K^+ is less than that from cells high in Na^+ (R. M. Johnstone, unpublished results).

Although the Na^+/K^+ ion gradient hypothesis, especially with respect to the role of the K^+ gradient, requires that the system be highly sensitive to K^+_o, (1) Kromphardt *et al.*[19] showed that if Na^+ is maintained at a constant level K^+_o has relatively little effect on uptake of glycine; (2) the data of Eddy and Hogg[20] show

TABLE I

CATION GRADIENTS AND GLYCINE ACCUMULATION AT STEADY STATE

In Expt 1, the cells were incubated for 60 min with stirring at 0–4 °C in K^+-free medium. Then the cells were centrifuged and suspended in 100 mM Na^+, 10 mM K^+, 50 mM choline chloride or 100 mM Na^+ + 60 mM K^+. Glycine was added to give a concentration of 0.1 mM and incubation continued at 37 °C. In Expt 2, there was no change of medium between the preincubation and actual experimental period. Cells were brought to steady state conditions by incubating for 60 min at 37 °C in a medium of 100 mM Na^+, 10 mM K^+, 50 mM choline chloride or a medium in which KCl replaced the choline chloride. After 60 min, sufficient glycine was added to make the final concentration 0.1 mM. HEPES buffer was used throughout. Samples were taken at intervals from 30 s to 60 min. The values for glycine uptake and ion distributions presented in this table are those after 60 min incubation in Expt 1 and 15 min in Expt 2. In both cases the systems had reached steady state with respect to the distributions of ions at the times reported above. Glycine distribution with high K^+ had reached at least 80% of the steady state value in both experiments at the times reported. In Expt 2 the intracellular ion concentrations reported are those that prevailed throughout the experimental period. In Expt 1, the initial intracellular $[Na^+]$ and $[K^+]$ were: low K^+ —180 mM Na^+, 80 mM K^+; and high K^+ —172 mM Na^+, 91 mM K^+. At 30 min the concentrations reported in the table were attained.

Expt	$[K^+]$ *Cellular* / *Extracellular* (*mequiv/l*)	$[Na^+]$ *Cellular* / *Extracellular* (*mequiv/l*)	*Potential energy from gradients* $2.3\ RT\ log\ [Na_o/Na_i \times K_i/K_o]$ (*cal/mole*)	*Glycine accumulation at steady state* G_i/G_o (*nmoles*)	*Theoretical energy required* $2.3\ RT\ log\ G_i/G_o$ (*cal/mole*)
1	[128]/[10]	[77]/[100]	$2.3\ RT\ \log\ 16.8 = 1740$	1400/100	1630
	[139]/[60]	[82]/[100]	$2.3\ RT\ \log\ 2.8 = 635$	1200/100	1530
2	[160]/[10]	[36]/[100]	$2.3\ RT\ \log\ 45.0 = 2350$	1360/100	1600
	[173]/[60]	[33]/[100]	$2.3\ RT\ \log\ 8.7 = 1300$	1030/100	1440

TABLE II

GRADIENT ENERGY AND ENERGY REQUIRED FOR GLYCINE ACCUMULATION

Data compiled from several experiments where glycine uptake at steady state exceeded that predicted from the calculated gradient energy. Experiments selected are those with 50–60 mM K^+ in medium. All values are from steady-state data using glycine concentrations of 0.1 mM. The average accumulation ratio based on nine experiments was 12.5 ± 1.8 (S.D.) with 10 mM K^+_o, and 11.4 ± 1.5 (S.D.) with 50–60 mM K^+_o.

Potential energy available in $Na^+ + K^+$ *gradients* (*cal/mole*)	*Energy required for glycine accumulation at steady state* (*cal/mole*)
1270	1480
1130	1340
845	1540
745	1400
700	1560
630	1550
440	1400

little inhibitory action of K^+_o unless the Na^+_o concentration is markedly reduced and K^+_o elevated; (3) the data of Schafer and Heinz[13] show that, in absence of ouabain, marked inhibition of uptake of α-aminoisobutyric acid (or "reversed" uptake) occurred only at very high K^+_o and low Na^+_o. Thus, the question whether the reduced uptake of α-aminoisobutyric acid is due to a smaller K^+ gradient or to an inhibition by high extracellular K^+ is not clear.

Our data show that, if K^+ is elevated to 60 mM but Na^+ maintained at 100 mM, there is little inhibitory effect of K^+ except on the initial rate of amino acid uptake.

DISCUSSION

The data in this communication center around the problem of the contribution of the K^+ gradient energy to accumulation of glycine. Although we would hesitate to state categorically that there is no contribution of energy from this source for glycine transport (or indeed from other ion gradients as well), the ion gradients are not a major source, that is, in excess of 10–20 %.

In this series of experiments the Na^+ gradient was small but the external Na^+ was maintained at a sufficiently high level to sustain amino acid accumulation. By working under conditions of a reduced Na^+ gradient, the contribution of the K^+ gradient energy was assessed more directly. Examination of the data in Table I and Fig. 1 shows that a reduction of the gradient energy from approx. 1700 cal/mole to 500–650 cal/mole can be obtained by elevating extracellular K^+ from 10 to 60 mM. This reduction of the available energy in the K^+ gradient does not markedly reduce glycine accumulation. (Indeed, the energy requirements for glycine accumulation at 37 °C cited in Table I do not vary by more than 200 cal/mole.) Since the maximum available gradient energy is nearly equivalent to that required for glycine accumulation (Fig. 1), it follows that most of the K^+ gradient energy is not coupled to glycine accumulation. Earlier Hempling and Hare[21] also concluded that the K^+ gradient is not a major source of energy for glycine accumulation in these cells. If, as suggested by Reid and Eddy[11], only a fraction of the K^+ gradient energy is used, the usable energy is still reduced by approximately 40 % when external K^+ is elevated. It may also be noted that with a normal K^+ gradient and 100 mM Na^+_o as well as a small Na^+ gradient, the efficiency of energy coupling of the ion and organic solute fluxes would have to be greater than 70 %, assuming that a 1:1 relationship exists between ion and glycine fluxes. This coupling would therefore have to be more efficient than that in oxidative phosphorylation.

The major evidence which suggests that the cation gradients are a source of energy for accumulation of organic solutes is the fact that organic solutes can be transported against their concentration gradients if ion gradients are provided in the apparent absence of metabolic energy[10,16,22], and that, under these conditions, accumulation is proportional to the gradient. However, few experiments have been conducted to determine whether the rate of ATP production under such circumstances is truly negligible or whether the steep ion gradients used in ATP-depleted cells permit some ATP synthesis by reversing the Na^+ pump. The latter has been shown to occur experimentally in red cell ghosts[23,24]. Preliminary experiments (R. M. Johnstone, unpublished results) with Ehrlich ascites cells indicate that in cells depleted

of ATP the presence of a "normal" inward Na^+ gradient *plus* an "outward" K^+ gradient results in the incorporation of ^{32}P into organic phosphate which is 2–5 times greater than in the absence of ion gradients.

Although our data are inconsistent with the conclusion that cation gradients provide energy for accumulation of amino acids in Ehrlich ascites cells, this does not imply that the asymmetric distribution of the cations does not play a role in amino acid accumulation. Much of our data are consistent with the following scheme.

(1) On the external surface of the membrane the carrier protein has a very high affinity for Na^+ relative to that for K^+. Na^+ stimulates amino acid transport whereas K^+ inhibits by altering the K_m values for the solute. But unless Na^+ is very low or K^+ high ($>$ 50 mM) the presence of K^+ does not interfere with the stimulatory effect of Na^+ on amino acid accumulation. In other systems the relative affinities for Na^+ and K^+ may be different so that K^+_o is inhibitory at lower concentrations ($>$ 20–30 mM).

(2) On the inside of the cell, a different situation obtains. Here the relative affinity of the amino acid transport system for K^+ is much greater than for Na^+. As on the external surface, the presence of K^+ on the carrier decreases amino acid–carrier association.

(3) There is no reason *a priori* to assume that the ratio of affinities for Na^+/K^+ on the outside are the same as those on the inside. Indeed, Charalampous[25] has shown in cultured cells that so long as the cellular K^+ is maintained above certain levels (30 mM or more) amino acid transport is maintained and that, if cell K^+ is low, the system gradually loses activity. Thus, we propose that so long as K^+_i remains above the critical level, it will compete effectively with Na^+_i. If the affinity of the solute carrier for Na^+ and K^+ were the same on both sides of the membrane, the concept of ion gradients would have to be invoked to explain the asymmetric distribution of transported organic solutes. The present proposal suggests that the system is asymmetrical with respect to the affinities of the transport system for the monovalent cations, not merely asymmetric because the relative ion concentrations are different on the two sides of the membrane.

(4) The cation gradients do not supply a major fraction of the energy required for accumulation of solute against a gradient. Cellular ATP (or a similar compound) is required. Cellular ATP in Ehrlich cells decreases the K_m for methionine uptake[16] whereas in mouse pancreas, K_m and V for an amino acid transport system are modified by ATP[17]. A possible role for ATP would be to maintain the asymmetry of the system with respect to the relative affinities for Na^+ and K^+ on the two sides of the membrane. ATP would thus appear to enhance the effects of the asymmetric distribution of cations and accumulation of organic solute would continue despite "reversed gradients"[13,14,16,17,26] so long as sufficient Na^+ were present externally and sufficient K^+ internally. This type of scheme would explain (a) amino acid transport in the absence of ion gradients in cells containing ATP as well as in those low in ATP but with normal ion gradients, (b) the lack of effect of high cell Na^+, and (c) the apparent relationship between the magnitude of the ion gradients and amino acid accumulation when the ion distributions are nearly completely reversed, particularly in cells low in ATP.

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