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THE EFFECT OF REVERSAL OF Na^+ AND K^+ ELECTROCHEMICAL POTENTIAL GRADIENTS ON THE ACTIVE TRANSPORT OF AMINO ACIDS IN EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. The net uptake of α-aminoisobutyric acid (AIB) in Ehrlich ascites tumor cells has been studied under a variety of transmembrane concentration gradients of Na^+, K^+ and AIB itself.

2. Before the transport measurements, the cells were prepared in such a way as to abolish or minimize exchange of extracellular AIB with endogenous amino acids, although the incubation with AIB was shown to have no significant effect on the intracellular concentrations of endogenous amino acids, as determined by an amino acid autonanalyzer.

3. The results are in agreement with the ion gradient hypothesis, in that the uptake of AIB rises in proportion to the increase of the parallel electrochemical potential gradient of Na^+ and the antiparallel electrochemical potential gradient of K^+.

4. Reversal of the net AIB flux was observed only after very drastic inversion of the above-mentioned gradients, *i.e.* if the driving forces were on the order of -4000 joules·mole^{-1} in opposition to the inward movement of AIB.

5. We have concluded that an additional driving force of at least 4000 joules·mole^{-1} (approximately 950 cal·mole^{-1}) must be present in addition to the electrolyte gradients. This unexplained driving force may be provided by direct coupling of amino acid transport to cellular metabolism, although alternative explanations such as an unequal distribution of alkali metal ions in the cellular space, *e.g.* between the nucleus and cytoplasm, cannot be completely excluded at this time.

6. In the presence of 0.5 mM ouabain, the deficit in driving force is reduced to 2240 joules·mole^{-1}, but it is not abolished.

INTRODUCTION

Over the past decade a considerable number of observations have suggested a coupling between non-electrolyte and Na^+ transport in a variety of tissues. This work has been well summarized in a recent review by SCHULTZ AND CURRAN[1]. With regard

Abbreviation: AIB, α-amino-isobutyric acid.

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to amino acids, the most frequent finding is that in many cells the active accumulation of these non-electrolytes requires the presence of Na^+ in the bathing medium, or in the mucosal medium for those epithelia actively transporting amino acids[1-5]. This requirement can be largely attributed to a specific activating effect of extracellular Na^+ on the unidirectional influx of these amino acids. In contrast, an increase in the intracellular Na^+ does not affect the unidirectional influx, but reduces the net uptake of amino acid into the cell[6]. Thus, the cellular Na^+ appears to activate amino acid efflux, just as the extracellular Na^+ activates influx. Consequently, it is not the mere presence of Na^+ but rather its gradient across the cellular membrane that is decisive for the activating effect. It has been postulated that the electrochemical potential gradient of Na^+, and possibly the opposing one of K^+, provide energy for active amino acid accumulation, Na^+ *via* co-transport and K^+ *via* countertransport with regard to the amino acid. This "ion gradient hypothesis" is strongly supported by the following observations: (1) The entry of amino acids into the cell is associated with a net gain of Na^+ and a net loss of K^+ (refs. 2, 7). In some cases an increment in Na^+ influx has been shown to be stoichiometrically related, usually in a one-to-one ratio, to the amino acid influx[8-11]. (2) Inversion of the Na^+ and K^+ electrochemical potential gradients, either by varying the distribution of these ions between the cell and the medium, or by altering the electrical potential across the cellular membrane, may stop or even invert the net movement of amino acids[10,12,13]. (3) In complete metabolic inhibition, *e.g.* by NaCN, or with extremely low levels of high energy phosphates, active transport of amino acids into or out of the cell can be demonstrated by maintaining appropriate electrolyte gradients[10,13,14]. To the extent that the driving force for the active amino acid transport is derived from the electrochemical potential gradients of the electrolytes, the transport of the non-electrolyte is only "secondarily active", because it depends on the production and maintenance of the ion electrochemical potential gradients. The question now becomes whether the amino acid transport is secondarily active in its entirety, *i.e.* whether all the energy required for active transport stems from the electrolyte gradients, or whether there is, in addition, a "primary" or direct coupling between amino acid active transport and metabolism. EDDY[14] did in fact report that glycine distribution ratios in NaCN-treated ascites cells maintained with normal electrolyte gradients were only 30 % of those seen for normal respiring cells under otherwise similiar conditions.

JACQUEZ AND SCHAFER[12] have investigated whether the ion gradients are the sole energy source by measuring α-aminoisobutyric acid (AIB) uptake in Ehrlich ascites cells with either the Na^+, or both the Na^+ and K^+ electrochemical potential gradients reversed. Although the AIB influx was still active even when both gradients were reversed, this could also result if AIB were driven inward by counterflow in exchange for endogenous intracellular amino acids. The levels of endogenous amino acids in Ehrlich cells have been shown to be high enough to make such a mechanism feasible[15]. In the experiments presented here, we have attempted to exclude the possibility of such an exchange by initially preloading the cells with a high level of AIB, in the expectation that any cellular amino acid capable of exchanging with AIB would be "washed-out" of the cell, and in order to permit wide variations and even reversal of both ion and AIB concentration gradients in the final incubation simply by adjusting the AIB, Na^+ and K^+ concentrations in the medium. The results, although they show that an active efflux of AIB could be produced when both the ion electro-

chemical potential gradients were drastically reversed, also show that these gradients, as determined from cellular and extracellular ion concentrations, fall far short of fully accounting for the amino acid active transport in this cell.

In addition, analyses of endogenous amino acid pools with an automatic amino acid analyzer show that the levels of intracellular amino acids were not altered by preloading the cells with AIB, a finding which appears to exclude the possibility that exchange diffusion contributes appreciably to the active transport of AIB.

METHODS

Composition of buffer solutions

The standard medium used was a Krebs Ringer phosphate buffer, which we shall refer to as Na-buffer, of the following composition: 124.6 mM NaCl, 7.9 mM KCl, 1.9 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10.0 mM Na_2HPO_4. This buffer, as all the buffers described below, was titrated to pH 7.4 with a small amount of 0.3 M HCl.

In some experiments a buffer in which Na^+ was replaced by choline was used. This buffer is referred to as choline-buffer, and contained 124.6 mM choline chloride in place of NaCl, and 10.0 mM K_2HPO_4 in place of Na_2HPO_4. Both of these buffers had an osmolality of 280 mosmoles$\cdot l^{-1}$. Other experiments employed a hypertonic buffer (400 mosmoles$\cdot l^{-1}$) in which K^+ was the major cation. This buffer is referred to as K-buffer and was composed of: 198 mM KCl, 1.9 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10.0 mM K_2HPO_4. In addition, all buffers contained albumin (1 %, w/v).

Ouabain was added to these buffers when required by first dissolving 364 mg of ouabain in 10 ml of absolute ethanol. 1 ml of this stock was then added to each 100 ml of buffer to give a final ouabain concentration of 0.5 mM.

Collection of the cells

The Ehrlich ascites tumor used for these experiments was a diploid line regularly maintained in this laboratory. Seven days after inoculation the mice were killed by cervical dislocation. The peritoneum was then exposed, and the ascites was removed by aspiration with a syringe. The ascites was placed in 20 ml of Na-buffer containing 0.5 mg heparin chloride. The ascites was almost always free of visible blood, but whenever any was present the ascites was discarded. Approximately 40 ml of ascites were collected per experiment, and were then run through a piece of nylon hose and centrifuged, after which the cells were ready for further preparative procedures.

Preparation of the cells for the test incubation

At this stage the cells were loaded with AIB, and were then exposed to a sequence of cold, one-half isotonic incubations, and cold, isotonic incubations. This sequence, described in detail below, was undertaken merely to prepare cells containing AIB at a concentration of 2–6 mM, and having high intracellular Na^+ and low intracellular K^+ concentrations. Cells so prepared were then used for the final incubation from which the important data derives.

The pellets of Ehrlich cells, after one washing with Na-buffer (above), were resuspended 1:20 with a solution of 2 mM AIB (labeled with ^{14}C) in Na-buffer. This suspension was incubated for 30 min at 37° with shaking. After this incubation, two 4-ml samples were removed to test tubes, and the remaining suspension was centri-

fuged and resuspended 1:10 in a cold (0–5°) solution consisting of 50 % Na-buffer and 50 % distilled water (cold shock). After 10 min at 0–5° this suspension was centrifuged and the pellets were resuspended 1:10 in full isotonic Na-buffer and were allowed to stand at 0–5° for 10 min (cold incubation). From this suspension, two 4-ml samples were taken, and the remaining suspension was centrifuged. The sequence of cold shock–cold incubation was repeated exactly as described above, except that the suspension of cells for the last cold incubation was 1:4 in Na-buffer. When ouabain was used in an experiment, it was present in the cold shock solutions at 0.25 mM, and in the cold incubation solutions at 0.5 mM, but was not present in the first incubation medium.

Final incubation procedure

After the last 10 min period at 0–5°, two 1.5-ml samples were pipetted from the suspension into two test tubes each containing either 3 ml K-buffer or 3 ml choline-buffer, depending on which was used for the final incubation. These samples provided a measure of the AIB, Na^+ and K^+ concentrations present in the cells at the beginning of the final incubation, which are later referred to as the initial concentrations. From the remaining cold incubation suspension, eight 1.5-ml portions were pipetted onto the bottoms of eight 25-ml incubation flasks with detachable side-arms. Each of the side-arms contained 3 ml of an AIB solution in choline-buffer or (in separate experiments) K-buffer, or in a few cases, hypertonic Na-buffer. Four different extracellular AIB concentrations were used within each experiment, and there were two replicates of each concentration. The [^{14}C]AIB came from the same original stock solution as did the AIB used for loading. Therefore, the [^{14}C]AIB inside the cells had the same specific activity as that finally applied extracellularly.

The sidearm solutions and the cell suspensions in the 8 flasks were then all mixed simultaneously at room temperature, and were immediately placed in a water bath at 37°. These suspensions were incubated with shaking for approx. 3 min, and were then tipped into the sidearms. The side arms were quickly removed and centrifuged at 0–5°. Subsequent handling of the sidearms and other samples is described below. Mixing of the sidearm solutions with the flask suspensions produced a suspension with approx. 40 mequiv·l^{-1} NaCl (derived from the 1.5 ml cell suspension) and 170 mequiv·l^{-1} KCl when K-buffer was used in the sidearms, or 14 mM KCl when choline-buffer was used. Since the cells had high intracellular Na^+ and low intracellular K^+ concentrations, the Na^+ concentration gradient across the cell membrane was reversed with choline buffer, and both the K^+ and Na^+ concentration gradients were reversed when K-buffer was used in the sidearm solutions. The AIB concentrations in the sidearms were chosen so as to produce the following approximate concentrations after mixing: 3.5, 5.3, 7.0 and 8.5 mM. These concentrations bracketed the initial intracellular AIB concentration, thereby providing samples in which initially the intracellular AIB concentration exceeded the extracellular and *vice versa*.

Analytical procedures

All samples, both in the side-arms and test tubes (initial samples), were centrifuged immediately after being taken. The supernatants were poured off and saved for analysis. The tubes containing the pellets were inverted over gauze for 20 min, and the walls of the test-tubes or side arms were then dried to within 2 mm of the pellet by

using a tissue wound around a pair of forceps. The sample tubes and side arms containing the pellets were then weighed (wet weight), frozen, and lyophilized overnight. The following morning the sample tubes and sidearms were reweighed (dry weight), and the pellets were removed with a spatula, each to a separate 10-ml glass homogenizer vessel, and the pellets were suspended in 3 ml distilled water. The suspensions were allowed to stand for 2 h at room temperature, and were then centrifuged to sediment cell debris. From the extract, 0.25 ml were diluted with 4 ml distilled water for flame photometric analysis and 0.5 ml were pipetted directly into scintillation vials along with 20 ml of toluene–ethanol scintillation fluid.

From each of the original supernatants, 0.1 ml were pipetted into test tubes and diluted with 10 ml water. This solution was used for the flame photometric analysis, and 0.5 ml of the supernatant was put into scintillation vials and handled as pellet samples.

Na^+ and K^+ concentrations were determined on a Netheler and Hinz "Eppendorf" flame photometer (propane gas) by comparison to standard solutions with the same Na^+ to K^+ ratios.

[^{14}C]AIB activity was determined using a Packard Tri-Carb Model 574 Liquid Scintillation Counter. A channels ratio procedure was employed to ascertain that quenching was equal among all samples. Supernatant and intracellular AIB concentrations were then computed by methods previously described[16]. The concentrations of AIB, Na^+ and K^+ are expressed as mM or mequiv·l^{-1}, although they are in reality mmoles or mequiv per kg intracellular water.

In some experiments, intra- and extracellular chloride concentrations were determined by reading the same dilutions prepared for flame photometry on an Aminco-Cotlove Chloride Titrator, and comparing to standard solutions.

In two experiments no labeled AIB was used and samples taken at various stages of the preparative procedure were prepared for analysis of endogenous amino acids as described below. In addition, another sample was taken from cells of the same batch which were incubated for 30 min at 37° without AIB present. Pellets of samples were extracted as described above; cell debris was centrifuged down. The supernatant extracts were then incubated for 5 min at 100° to precipitate proteins. This suspension was centrifuged, and the resulting supernatant was frozen and lyophilized. The residue after lyophilizing was dissolved in an amount of water appropriate to give good readings when the extracts were analyzed on a Technicon Automatic Amino Acid Analyzer.

Statistics

The use of replicates for all samples taken allowed us to estimate the random error involved in the experimental and analytical procedures. For the determination of intracellular and extracellular AIB, the mean coefficients of variation were 2.01 % and 0.97 %, respectively. For Na^+ and K^+ intracellular and extracellular concentrations the mean coefficients of variation were 2.66 % and 1.78 %, respectively. Most values in RESULTS are the averages of duplicates with the range appended.

Biochemicals and labeled compounds

α-Amino[1-^{14}C]isobutyric acid ([^{14}C]AIB) was obtained from the Radiochemical

Center, Amersham in 0.5 mC vials with a specific activity of 44.2 mC/mmole. This solid material was dissolved in 2 ml distilled water. To prepare a stock solution for an experiment, 0.2 ml (5 μC) of the radioactive solution were combined with 82.48 mg of unlabeled AIB and made to 10 ml with K-buffer or choline-buffer to give a solution of 80 mM AIB with [^{14}C]AIB present with a specific activity of 6.25 μC/mmole. All AIB-containing solutions were then made up by diluting this stock solution with the appropriate buffer solution.

Unlabeled AIB (extra pure grade) and pure beef albumin were obtained from Serva, Heidelberg. Ouabain and all other amino acids were obtained in extra pure grade from Merck, Darmstadt.

RESULTS

Intracellular ion and AIB concentrations after preparative procedures

Table I gives the intracellular concentrations of AIB, Na^+ and K^+ after the preparative procedures described in METHODS. The 30-min incubation with 2 mM AIB produced intracellular AIB concentrations which always fell in the range 19–25 mM, which we have found to be approximately the steady-state concentration reached even after longer incubations in 2 mM AIB. Na^+ and K^+ concentrations after loading fell into the range which can be regarded as normal for Ehrlich ascites tumor cells. The cold shock and cold incubation progressively reduced the AIB and K^+ concentrations while increasing the Na^+ concentration. At the end of these procedures (Stage 3 in Table I), the Na^+ and K^+ intracellular concentrations were reversed in regard to their normal relation, *i.e.* intracellular Na^+ was quite high, and the K^+ concentration was less than that of Na^+. Therefore, the Na^+ concentration gradient was reversed when the cells were suspended in choline-buffer after such preparation, and both the Na^+ and K^+ concentration gradients were reversed by suspension in K-buffer. Also, various AIB concentration gradients were produced by using AIB concentrations in the resuspending medium which were both higher and lower than the intracellular concentration.

TABLE I

INTRACELLULAR CONCENTRATIONS OF AIB, Na^+ AND K^+ AFTER VARIOUS STAGES OF THE PREPARATIVE PROCEDURE

Samples for analysis were taken after the procedures listed. Each stage is part of a sequence, therefore, cells at any stage have already undergone all preceding treatment. Concentrations are given in terms of the range observed for all experiments.

Procedure	*Intracellular concentration*		
	AIB (*mM*)	Na^+ (*mequiv*·L^{-1})	K^+ (*mequiv*·L^{-1})
1. 30 min incubation with 2 mM AIB at 37°	19–25	20–40	140–170
2. 10 min incubation in half isotonic medium followed by 10 min incubation in full isotonic medium, both at 0°	8–18	80–110	60–90
3. Repeat half isotonic–full isotonic incubation sequence	3–14	90–130	50–90

Intracellular levels of endogenous amino acids

One of the purposes of loading the cells with AIB was to "wash-out" any amino acids or other substances capable of exchanging with AIB so as to rule out the exchange contribution to the AIB movements measured subsequently. The results of our amino acid analyses are presented in Table II. Preincubation of the cells for 30 min at 37° with or without AIB led to a slight decrease in the concentrations of most amino acids. Any increases were not statistically significant. In general, the overall concentration pattern of the amino acids in "fresh" cells (Column a) agreed with those obtained by OXENDER[15], except that the concentrations we obtained were, as a rule, higher. The important comparison is between cells incubated for 30 min without AIB (column b) and those incubated with 2 mM AIB (Column c). If AIB were exchanging with endogenous amino acids, one would expect to find a reduction in the concentration of one or more endogenous amino acids in cells preloaded with AIB. The above comparison shows that no amino acid concentrations are significantly altered by the AIB loading.

Column d in Table II shows the amino acid concentrations after loading followed by the cold-shock and cold-incubation procedures. Approximately one-third of the amino acids show a much lower concentration after these procedures. Those amino

TABLE II

INTRACELLULAR LEVELS OF ENDOGENOUS AMINO ACIDS

Samples of cells were prepared as described in METHODS. Analyses were performed on cells taken immediately after collection and one Na-buffer wash (a), a second group of cells from the same batch were then incubated for 30 min at 37° without AIB (b), and a third group was incubated for 30 min at 37° with 2 mM AIB (c). From this third group some of the cells were then exposed to the sequence of cold-shock, cold-incubation procedures described in METHODS. All concentrations are given as the mean of results from two separate experiments with the range appended, except for (b) in which only one determination was made.

Amino acid	*Intracellular concentration (mM)*			
	(a) washed once with Na-buffer	*(b) After 30 min incubation at 37° without AIB*	*(c) After 30 min incubation at 37° with 2 mM AIB*	*(d) After 30 min incubation at 37° with 2 mM AIB, then cold-shock procedure*
Taurine	2.88 ± 0.56	2.06	2.29 ± 0.12	1.65 ± 0.04
Threonine*	2.62 ± 0.32	2.77	2.90 ± 0.10	2.23 ± 0.17
Serine*	2.45 ± 0.25	2.11	2.15 ± 0.50	1.92 ± 0.12
Glutamate	5.68 ± 0.04	4.68	4.74 ± 0.36	3.56 ± 0.11
Proline	4.44 ± 1.22	2.55	2.25 ± 0.22	2.16 ± 0.10
Glycine	5.56 ± 0.08	5.96	6.16 ± 0.18	3.60 ± 0.20
Alanine	5.56 ± 0.05	4.97	5.23 ± 0.26	3.22 ± 0.06
Valine	1.36 ± 0.08	1.29	1.30 ± 0.05	1.14 ± 0.14
Cysteine–methionine**	0.52 ± 0.14	0.52	0.48 ± 0.01	0.56 ± 0.13
Isoleucine	0.70 ± 0.02	0.79	0.68 ± 0.04	0.70 ± 0.02
Leucine	1.38 ± 0.12	1.60	1.44 ± 0.01	1.53 ± 0.12
Tyrosine	0.70 ± 0.08	0.78	0.72 ± 0.04	0.82 ± 0.04
Phenylalanine	0.72 ± 0.07	0.95	0.88 ± 0.12	0.84 ± 0.01
Lysine	2.38 ± 0.01	1.93	1.94 ± 0.06	1.97 ± 0.03
Histidine	0.46 ± 0.09	0.63	0.58 ± 0.01	0.53 ± 0.03
Arginine	2.20	1.08	0.94 ± 0.08	1.15 ± 0.10

* In three chromatograms the threonine and serine peaks could not be separated. Concentrations in the chromatograms were estimated from the total peak.

** Cysteine and methionine peaks always fell together.

acids showing the greatest loss with the cold-shock procedures are the same ones that OXENDER[15] found to be altered the most by room temperature osmotic shock.

The lack of any evidence for an exchange of endogenous amino acids with AIB is supported by other experiments we have conducted. In these experiments we loaded Ehrlich cells to high (30–80 mM) intracellular concentrations with AIB, glycine, L-alanine, L-leucine, L-serine, or L-valine, and then measured AIB 1 min influxes in Na-buffer and choline buffer. In all cases there was either no significant change in the AIB influx or else a slight decrease in comparison to non-loaded cells in the same medium.

AIB uptake with a reversed Na^+ electrochemical potential gradient

After the cells were prepared as described in METHODS and Table I, they were resuspended, in five experiments, in a choline-buffer solution with about 40 mequiv·l^{-1} Na^+, thereby reversing the Na^+ but not the K^+ concentration gradient. The direction of AIB movement during a 3-min incubation with both inwardly and outwardly directed AIB gradients was then measured. The results of two such experiments are given in Table III, Part A. Because ion concentrations were measured after the final incubation period and the reversed Na^+ concentration gradient decreased monotonically throughout the incubation, these concentrations represent the minimal degree of reversal of the sodium ion concentration gradient which was present during the course of the final incubation. Simultaneous measurement of the chloride distribution gave a chloride equilibrium potential of -8.9 ± 0.2 mV, which we have used as an approximation of the transmembrane potential. If this is actually the transmembrane potential, then we have computed that the Na^+ electrochemical must have been reversed in all such experiments. In any case, a transmembrane potential at least as electronegative as —15 mV would have been required in order for the Na^+ electrochemical potential gradient to be just zero in those experiments without ouabain, and —23 mV would have been required in experiments with ouabain.

Table III also gives the intracellular AIB concentrations before ($[AIB]_i^o$) and after ($[AIB]_i^f$) the final incubation period. Any increase in the intracellular AIB concentration indicates movement of AIB into the cells. In all experiments the intracellular AIB concentrations increased, and in all cases the final intracellular AIB concentrations exceeded the corresponding extracellular concentrations by a large margin. Thus, in spite of the reversed Na^+ electrochemical potential gradient, AIB was actively transported into the cells. Under the above conditions the cells showed no significant swelling or shrinkage during the final incubation, as determined from the ratio of pellet wet to pellet dry weight, *i.e.* there was no net water movement that might explain the AIB concentration effect.

AIB uptake with reversed Na^+ and K^+ electrochemical potential gradients

In 22 experiments, all but 40 mequiv·l^{-1}, or in some cases 10 mequiv·l^{-1}, of Na^+ in the final medium was replaced by K^+. At least initially, this led to reversal of both ion concentration gradients. It was found that in order to maintain a reversed K^+ concentration gradient, a hypertonic medium which had approx. 190 mequiv·l^{-1} K^+ had to be employed. This hypertonic medium also prevented any significant swelling of the cells which is found with isotonic K^+ buffers.

Table III, Part B gives the results of representative experiments in which both

TABLE III

THE EFFECT OF Na^+ AND K^+ CONCENTRATION GRADIENTS ON THE UPTAKE OF AIB

The data in this table represent the results of only a sampling of many experiments performed. To avoid any question of selection on the basis of the results obtained, we have included only those experiments in which the chloride distribution was measured (except for Expt. 15 in which no chloride distribution was measured). None of the experiments in which this measurement was made are omitted. These experiments comprise one-third of all experiments later analyzed in Figs. 1, 2 and 3, and show no significant differences from the bulk of the data obtained. In all experiments, the cells were loaded with AIB and prepared for the final incubation as described in the text. The final incubation was for 3 min at 37° in choline-buffer (Part A) or K-buffer (Part B) with AIB present at the indicated concentrations in the extracellular medium, $[AIB]_e$. Ouabain was present in the bathing medium at 0.5 mM where indicated. The intracellular AIB concentration before the final incubation is designated $[AIB]_i^0$ and that after the final incubation $[AIB]_i^t$. For the ion concentrations, the subscripts i and e denote, respectively, the intracellular and extracellular concentrations at the end of the final incubation. The transmembrane potential (inside with respect to outside) was estimated from the chloride distribution in these experiments, and was -8.9 ± 0.2 mV for the experiments in Part A, and -3.6 ± 1.6 mV for the experiments in Part B.

Expt. No.	*Ouabain (0.5 mM)*	$\frac{[Na^+]_i}{[Na^+]_e}$ *(mequiv·l⁻¹)*	$\frac{[K^+]_i}{[K^+]_e}$ *(mequiv·l⁻¹)*	$[AIB]_e$ *(mM)*	$[AIB]_i^0$ *(mM)*	$[AIB]_i^t$ *(mM)*
A) Experiments in which the Na^+ concentration gradient was reversed						
23	—	56.53 ± 1.17 / 38.06 ± 0.71	102.69 ± 1.96 / 15.96 ± 0.28	3.51 ± 0.10 5.39 ± 0.09 7.10 ± 0.09 8.58 ± 0.10	14.03 ± 0.13	22.00 ± 0.00 22.73 ± 0.24 25.50 ± 0.36 26.43 ± 0.28
24	—	82.90 ± 2.38 / 47.90 ± 0.66	95.18 ± 1.06 / 15.15 ± 0.11	3.50 ± 0.01 5.23 ± 0.04 6.84 ± 0.04 8.61 ± 0.01	7.77 ± 0.03	16.63 ± 0.06 18.49 ± 0.15 19.72 ± 0.14 21.14 ± 0.22
26	+	101.54 ± 2.45 / 43.90 ± 0.82	75.74 ± 2.41 / 16.49 ± 0.14	3.83 ± 0.05 5.57 ± 0.09 7.24 ± 0.12 8.90 ± 0.05	8.70 ± 0.03	9.22 ± 0.01 11.40 ± 0.03 13.60 ± 0.09 14.70 ± 0.07
B) Experiments in which both the Na^+ and K^+ concentration gradients were reversed						
14	—	75.42 ± 2.33 / 43.89 ± 0.70	148.97 ± 4.18 / 162.86 ± 1.51	3.65 ± 0.07 5.31 ± 0.02 6.99 ± 0.06 8.53 ± 0.05	11.71 ± 0.38	11.37 ± 0.13 13.80 ± 0.09 15.20 ± 0.04 17.60 ± 0.01
19	—	63.19 ± 1.74 / 43.24 ± 1.06	152.50 ± 3.64 / 162.64 ± 1.55	3.65 ± 0.07 5.41 ± 0.03 7.12 ± 0.08 8.64 ± 0.09	8.73 ± 0.05	9.04 ± 0.08 10.92 ± 0.04 12.27 ± 0.06 13.71 ± 0.24
21	—	95.91 ± 2.19 / 40.06 ± 1.19	133.73 ± 2.44 / 149.40 ± 1.90	3.71 ± 0.01 5.81 ± 0.11 7.35 ± 0.02 9.00 ± 0.05	6.80 ± 0.13	7.25 ± 0.21 8.42 ± 0.20 9.29 ± 0.12 10.15 ± 0.37
15	—	111.65 ± 2.35 / 44.20 ± 0.68	131.10 ± 1.87 / 163.06 ± 2.08	3.68 ± 0.08 5.39 ± 0.04 7.19 ± 0.08 9.05 ± 0.04	6.65 ± 0.07	3.87 ± 0.00 5.11 ± 0.10 6.01 ± 0.02 6.81 ± 0.01
5	+	153.30 ± 1.81 / 43.67 ± 0.45	98.94 ± 1.65 / 152.28 ± 1.41	3.85 ± 0.01 5.53 ± 0.02 7.21 ± 0.06 8.88 ± 0.00	5.75 ± 0.04	1.77 ± 0.01 2.53 ± 0.11 3.14 ± 0.03 3.88 ± 0.00
42	+	141.38 ± 5.33 / 10.09 ± 0.77	135.43 ± 5.17 / 209.38 ± 1.02	3.89 ± 0.01 5.45 ± 0.05 7.40 ± 0.01 8.92 ± 0.02	5.75 ± 0.06	0.76 ± 0.18 1.01 ± 0.06 1.22 ± 0.13 1.34 ± 0.00

ion concentration gradients were reversed. Measurement of the chloride distribution gave -3.6 ± 1.6 mV as an estimate of the transmembrane potential. For any negative transmembrane potential, the K^+ electrochemical potential gradient must be reversed if the concentration gradient is reversed. In order for the Na^+ electrochemical potential gradient to be just zero would require a transmembrane potential more electronegative than —11 to —23 mV, depending on the particular experiment in question. In 15 experiments conducted in the absence of ouabain, both the Na^+ and K^+ electrochemical potential gradients were reversed throughout the final incubation period. Yet in all but three of the 15 experiments, AIB moved into the cells to such an extent that the final intracellular AIB concentration exceeded the extracellular concentration even when the AIB gradient was initially directed outward (*cf.* Table III, Part B, Expts. 14, 19 and 21). In the other three experiments without ouabain, one of which (Expt. 15) is given in Table III, the Na^+ and K^+ electrochemical potential gradients remained reversed to a much greater extent. In 6 of the 12 test situations in these three experiments, AIB moved *out* of the cells against a concentration gradient. However, in these three experiments (but only these), there was a significant degree of cell swelling. Thus, the decrease in intracellular AIB might be attributable to the net water influx and poor condition of the cells, rather than to active AIB extrusion. Changes in the AIB extracellular concentrations were too small in these particular cases to allow a firm conclusion to be made.

In seven experiments involving K-buffer replacement of Na-buffer in the bathing medium, ouabain was used in the preparative and final incubations. As seen from the results of Expts. 5 and 42 in Table III, Part B, the Na^+ and K^+ concentration gradients were reversed to a much greater extent than in comparable experiments without ouabain. In these experiments, AIB left the cells to such an extent that its final intracellular concentration was much less than the extracellular concentration. That is, as with Expt. 15, there was an apparent active transport of the AIB out of the cells whether the AIB concentration gradient was initially inwardly or outwardly directed. This occurred in seven experiments, and no active inward AIB transport was seen in any of the seven experiments in which ouabain was employed. In two of these experiments, we also resuspended some of the cells in a hypertonic Na-buffer medium for comparison with the results with the hypertonic K-buffer medium. In the presence of the resulting normally directed but reduced Na^+ and K^+ electrochemical potential gradients, it was seen that these cells were still capable of active inward AIB transport.

In summary, the results obtained for the experiments in which both ion electrochemical potential gradients were reversed showed that AIB could definitely be transported inward against its electrochemical potential gradient in spite of the reversals. However, when both ion gradients were strongly reversed such as seen in the presence of ouabain, active amino acid exit from the cell could be demonstrated.

DISCUSSION

We wish to examine the results presented with regard to the theory that the electrochemical potential gradient of Na^+ or a combination of the electrochemical potential gradients of Na^+ and K^+ provide the necessary energy for amino acid active transport in Ehrlich ascites tumor cells. EDDY[14] has convincingly demonstrated that

the Na^+ electrochemical potential gradient is the major determinant of glycine accumulation in NaCN-poisoned ascites tumor cells, and that there is a slight additional contribution from the K^+ gradient. However, because these electrolyte gradients were adequate to account for the accumulation only in inhibited cells, and not in respiring cells, these studies cannot exclude an additional energy contribution from cellular metabolism.

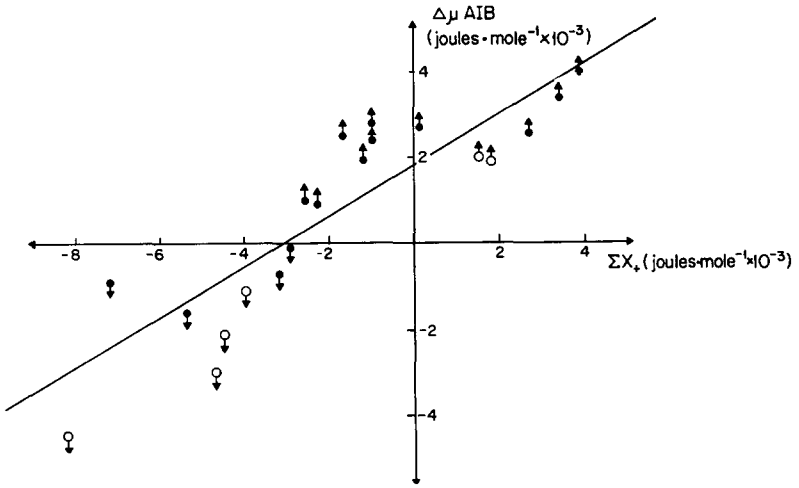

Fig. 1. The dependence of the chemical potential gradient of AIB on the combined Na^+ and K^+ electrochemical potential gradients. The AIB chemical potential difference is that calculated from the difference between intra- and extracellular concentrations of AIB after the final 3-min incubation. The arrow associated with each dot denotes whether this final AIB gradient is larger or smaller than the corresponding one just prior to the final incubation. ΣX_+ denotes the sum of the presumed driving forces, *i.e.* the electrochemical potential gradients of Na^+ and K^+ as described in the text. The data are taken from several experiments for those samples with an extracellular AIB concentration of 5–6 mM. Open circles represent experiments with and closed circles without ouabain.

The present results from normally respiring Ehrlich cells with altered ion electrochemical potential gradients agree with the hypothesis that the Na^+ and K^+ gradients contribute energy to the active accumulation of AIB in these cells. This conclusion is demonstrated by Fig. 1, in which the transient (3 min) chemical potential gradient of AIB is plotted as a function of the sum of the inwardly directed Na^+ and the outwardly directed K^+ electrochemical potential gradients. These electrochemical potential gradients were computed from the intra- and extracellular ion concentrations in all experiments conducted, and the transmembrane potentials were computed from the chloride distributions reported in Table III. The chloride distribution is the most convenient means of assessing the membrane potential, but we cannot ascertain how reliable this approximation is under conditions such as those employed in these experiments, in which the cation gradients were reversed. AULL[17] has shown by micropuncture techniques that the chloride equilibrium potential is nearly identical to the actual transmembrane potential for cells in a medium of normal composition. Furthermore, on the basis of more limited experimental evidence, it appears that the substitution of the less permeable cations, choline and K^+, for Na^+ in the bathing medium should make the potential less electronegative as we would predict from our calculated

TABLE IV

THE EFFECT OF THE K^+ ELECTROCHEMICAL POTENTIAL GRADIENT ON THE INFLUX OF AIB

The data is from experiments such as those in Table III in which the Na^+ electrochemical potential gradient was approximately the same, but in which the K^+ electrochemical potential gradient (X_K) showed wide variation. J_{AIB} is the 3-min influx of AIB per g dry wt. of ascites, measured at the lowest external AIB concentration in each experiment. The correlation coefficient for J_{AIB} plotted as a function of X_N (the Na^+ electrochemical potential gradient) was 0.41, whereas that for J_{AIB} plotted as a function of ΣX_+, the sum of the Na^+ and K^+ electrochemical potential gradients, was 0.85. To compute X_K and X_N, the transmembrane potential used was that computed from the chloride distributions as noted in Table III. Since the extracellular AIB concentrations were practically the same in all experiments listed, the AIB electrochemical potential gradient was not included in the summation of the gradients.

Expt. No.	X_N (*joules·mole*$^{-1}$)	X_K (*joules·mole*$^{-1}$)	ΣX_+ (*joules·mole*$^{-1}$)	J_{AIB} (μ*moles·g*$^{-1}$*·3 min*$^{-1}$)
14	−880	−801	−1681	−0.34
30	−756	+3438	+2682	+5.42
8a	−563	−769	−1333	+4.27
18	−550	−405	−965	−0.76
24	−584	+4023	+3439	+8.86
16	−688	−302	−990	−1.55
19	−385	−797	−1182	+0.31
10a	−225	−385	−610	+3.84
23	−117	+4028	+3911	+7.97
45	−1267	−1034	−2301	+0.82

chloride potentials[17]. Although these experiments had not been specifically designed to test for a contribution of the K^+ gradient, such a contribution is strongly suggested by the following. In Table IV a group of samples is listed in which the extracellular AIB concentration is rather constant at about 2.5 mM, and in which the electrochemical potential gradients of K^+ vary widely between −1000 and +4000 joules·mole^{-1} as compared to relatively moderate variations in the corresponding Na^+ electrochemical potential gradients. If the 3 min flux of AIB, J_A, is plotted *versus* the Na^+ electrochemical potential gradient, X_N, only a poor correlation is obtained (correlation coefficient, $r = 0.41$). On the other hand, if the same J_A values are plotted *versus* the combined driving forces ($X_N - X_K$, where X_K is the K^+ electrochemical potential gradient) as in Fig. 1, there is a good correlation ($r = 0.84$). Since most of the values plotted in Fig. 1 are transient, the tendency of their change during the final incubation is indicated by a small arrow attached to each experimental point. It is seen that all AIB chemical potential gradients corresponding to driving forces above −2500 joules·mole^{-1} are positive with rising tendencies. Hence, there is an active accumulation even with absent or negative, *i.e.* opposing, driving forces, which implies that there must be some driving force acting in addition to the electrolyte gradients. In order to estimate the magnitude of this extra driving force, we have stated the problem by means of the following theoretical development, which applies and extends a procedure described by RAPOPORT[18].

We assume that all the coupled fluxes of Na^+, K^+ and AIB can be treated as resulting from a single reaction:

$$\nu_A A_e + \nu_N Na_e^+ + \nu_K K_i^+ + \nu_S S_i \rightleftharpoons \nu_A A_i + \nu_N Na_i^+ + \nu_K K_e^+ + \nu_P P_i \qquad (1)$$

The subscripts e and i refer, respectively, to the extra- and intracellular phases. A is the amino acid, S and P the substrate and product, respectively, of a chemical reaction occurring inside the cell which may or may not be linked to the solute movements. ν_A, ν_N, ν_K, ν_S, ν_P, are the stoichiometric coefficients of the reactants AIB, Na^+, K^+, S and P, respectively. If the reaction is at or near equilibrium, the rate of the overall reaction, J_r, is proportional to the decrease in the overall free energy, $-\Delta G_r$, for the reaction:

$$J_r = L_{rr}(-\Delta G_r) \tag{2}$$

where L_{rr} is the coefficient of proportionality. It is fairly reasonable to assume that the reaction is indeed near equilibrium since the driving force for the chemical reaction is comprised of several coupled individual driving forces which are, at least in part, mutually opposing. That is, ΔG_r is composed of the individual electrochemical potential gradients of the solutes and the free energy change of the reaction proper:

$$\Delta G_r = \nu_A \Delta\tilde{\mu}_A + \nu_N \Delta\tilde{\mu}_N - \nu_K \Delta\tilde{\mu}_K + \nu_P \mu_P - \nu_S \mu_S \tag{3a}$$

where the electrochemical potential gradients of AIB, Na^+ and K^+ are taken as intracellular with respect to extracellular. As is usual in irreversible thermodynamics, the negative electrochemical potential gradients are replaced by positive driving forces, X_i, and the negative free energy change of the chemical reaction by the positive affinity, A_r (see footnote*), so that:

$$-\Delta G_r = \nu_A X_A + \nu_N X_N - \nu_K X_K + A_r \tag{3b}$$

The net flux of AIB may now be expressed as the sum of the flux due to the chemical reaction *plus* a parallel leak flux, $L_{AA}X_A$:

$$J_A = \nu_A J_r + L_{AA}X_A \tag{4a}$$

substituting:

$$J_A = \nu_A L_{rr}(\nu_A X_A + \nu_N X_N - \nu_K X_K + A_r) + L_{AA}X_A \tag{4b}$$

In view of previous observations on the leak rate in these cells[19], it seems reasonable to consider the leakage term to be negligible as compared to the coupled flux. Furthermore, we assume that one AIB is moved for every Na^+ moved inward and K^+ outward, *i.e.* $\nu_A = \nu_N = \nu_K$ (refs. 8, 14). Eqn. 4b then simplifies to:

$$J_A = \nu_A^2 L_{rr}\left(X_A + X_N - X_K + \frac{A_r}{\nu_A}\right) \tag{5a}$$

or,

$$J_A = \nu_A^2 L_{rr}(\Sigma X_{vec} + A_r/\nu_A) \tag{5b}$$

where ΣX_{vec} represents the sum of the vectorial driving forces other than the affinity. If we now plot J_A as a function of the vectorial driving forces, we should obtain a function which passes through the origin if no chemical reaction is involved ($A_r = 0$).

* It should be noted that in this development the affinity becomes a vector rather than the customary scalar dimensionality. This anomaly arises because the reaction has a definite spatial orientation with regard to the cell membrane as implied by the nature of the coupling. Such a system is distinctly anisotropic, and one could not expect the Curie theorem to hold. Furthermore, the directionality of the affinity also implies that the rate of the reaction, J_r, must also be a vector.

If A_r is positive, then the function should be shifted to the negative side and intersect with the x-axis at a value of ΣX_{vec} which is equal and opposed to the effective chemical driving force (A_r/ν_A). The result is seen in Fig. 2. The plotted values are well correlated with two linear regression lines ($r = 0.88$). Since the linearity of the function has doubtful significance in view of the assumptions made, the meaning of the slope will not be discussed here. The intersections of the regression lines with their

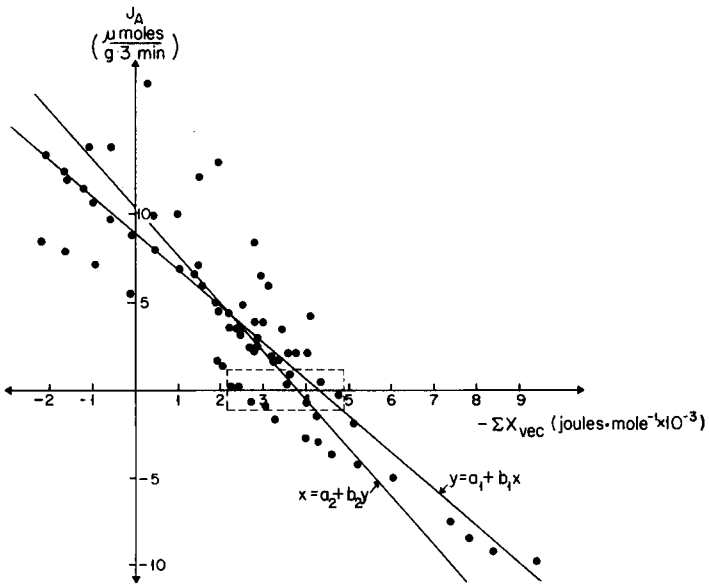

Fig. 2. The net influx of AIB as a function of the sum of the vectorial driving forces. J_A is the net flux per g dry weight over the final 3-min incubation period. ΣX_{vec} is the sum of the vectorial driving forces for AIB, represented by the electrochemical potential gradients of Na^+, K^+ and AIB itself, *i.e.* $X_A + X_N - X_K$. All the data are from those experiments in which no ouabain was used. The two lines represent the least squares regression of y on x ($y = a_1 + b_1x$, $r = 0.88$), and of x on y ($x = a_2 + b_2y$, $r = 0.88$). The dotted lines enclose points for which J_A is approximately zero.

respective axes, however, show a highly significant difference from zero, and are considered important in this context. The x-intercept for the regression of x on y is 3790 ± 148 joules·mole^{-1} ($P < 0.001$), and the y-intercept of the regression of y on x is 8.95 ± 0.55 μmoles·g^{-1}·3 min^{-1} ($P < 0.001$). From the graph, two things are obvious: (1) When the overall vectorial driving force (ΣX_{vec}) is zero, there is still a substantial transport of AIB into the cell (approximately 9 μmoles g^{-1}·3 min^{-1}). (2) The net flux of AIB is zero or negative only if the overall vectorial driving force is strongly negative, on the order of —4000 joules·mole^{-1} (or approximately —950 cal·mole^{-1}). Hence, there must be an additional driving force of this order of magnitude but in a direction opposite to the sum of the vectorial driving forces. Such a conclusion would be consistent with the assumption that AIB transport is directly coupled to metabolism, in other words, that A_r/ν_A is finite and of the order of 4000 joules·mole^{-1}, In order to ascertain this assumption, we must examine all other alternative possibilities such as:

(1) There may be an additional solute involved whose electrochemical potential

gradient provides a driving force for AIB transport. However, no evidence has ever been reported that the transport of amino acids is linked to any gradients other than those of the two alkali ions.

(2) The stoichiometric coefficient of AIB transport (ν_A) could be smaller than that of the ion movements, which would indicate that more than one ion is transported or exchanged for each AIB molecule. Apart from the fact that the stoichiometric ratio between AIB and Na^+ influx has been shown to be unity[8], the following considerations appear to contradict this alternative. Let us, for instance, assume that the movement of two Na^+ and two K^+ is linked to the transport of one AIB molecule, then, ν_K and ν_N would be $+2$. Therefore, the electrochemical potential gradient of each ion would have to be multiplied by two in Eqn. 5a. An inspection of those points in which transport is approximately zero (enclosed with dotted lines in Fig. 2) shows that for all of them the electrochemical potential gradients of both Na^+ and K^+ are inverted. Hence a higher ν_N and/or ν_K would make the missing force appear even greater than suggested above. The situation would be similiar if the coefficient of AIB (ν_A) were greater than ν_N and ν_K, since in the above-mentioned samples the chemical potential difference of AIB is also positive. It follows that a higher stoichiometric coefficient of any of the three solutes could not account for the observed deficit in the driving force.

(3) The effective vectorial driving force ($-\Sigma X_{vec}$) could be considerably greater, *i.e.* less negative, than the values plotted in Fig. 2. This supposition would imply that one or more of the individual electrochemical potential gradients, as derived from the distribution ratios and chloride equilibrium potentials, was incorrect, *i.e.* not truly representative of the driving force effective across the membrane. For instance, AIB could be partially bound to intracellular sites so that the free AIB inside the cell would have a lower chemical potential than had been assumed. Although the binding of AIB, Na^+ and K^+ has not been completely eliminated as a possibility, and in fact there is evidence that as much as 65 % of the sodium in muscle, brain and kidney may be complexed by tissue macromolecules[19], such a possibility seems unlikely in view of extensive evidence that the intracellular amino acid is freely dissolved[20], and it would not be consistent with the finding that during complete metabolic inhibition the AIB accumulation is almost exactly matched by the driving electrochemical potential gradients of Na^+ and K^+ (ref. 14). Another more serious alternative has been suggested by EDDY[14]. His alternative explanation is that, due to a highly active Na^+–K^+ pump, an unstirred layer is in effect maintained near the inner face of the membrane. This unstirred layer would contain a lower Na^+ and a higher K^+ concentration than the overall intracellular space[14]. As a consequence, the effective driving forces attributable to the ion gradients would be much larger than those derived from the overall distribution ratios. If we assume that the electrochemical potential gradient of AIB is correct, and that the unexplained driving force of about 4000 joules·mole^{-1} reflects errors in both the Na^+ and K^+ gradients to equal extents, we can postulate that the effective K^+ concentration in this region must be double that in the cellular bulk phase. The existence of such a region of lower Na^+ and higher K^+ concentrations, produced by an active ion pump would indeed account for the missing driving force without making the assumption of a direct metabolic coupling. However, this hypothesis critically depends upon how long such a region, which is of necessity transient, might exist before diffusion inside the cell would lead to a steady state with an even distribution of Na^+ and K^+ throughout the cellular space. As will be shown elsewhere[21] such a

region can hardly be maintained for a sufficiently long time without an additional energy barrier inside the cell. The diffusion coefficients for the alkali metal ions inside these cells are not known, but if they are comparable to those shown for the giant squid axon[22] or muscle[23], *i.e.* of the same order of magnitude as those in free solution, complete equilibration inside the cell should take only a fraction of a second. A delay of this equilibrium for only a few minutes would require the corresponding diffusion coefficients be five orders of magnitude lower than in free solution, which appears very unlikely. On the other hand, there is no evidence whatsoever of an additional energy barrier in the periphery of the cell, near the cell membrane. One may, however, modify EDDY's[14] hypothesis by invoking the nuclear membrane as such a barrier, and by assuming that the Na^+ concentration is lower and the K^+ concentration higher in the cytoplasm than in the nucleus, as has been shown for liver cells[24]. Since the nucleus of the Ehrlich cell is relatively large, the overall intracellular ion concentrations may be largely determined by their intranuclear concentrations. In order for the differential electrolyte concentration between nucleus and cytoplasm to account for the extra AIB accumulation, the amino acids, unlike the alkali metal ions, would have to distribute rather readily over the entire intracellular space. In other words, one has to postulate that the same barrier which bars the passage of Na^+ and K^+ is freely permeable to AIB. In order to maintain the concentration difference between the nucleus and cytoplasm for a sufficiently long time, the barrier would have to be rather tight. As will be shown elsewhere[21], the permeability coefficients of Na^+ and K^+ must be less than 10^{-7} cm·sec^{-1} if the half-time of equilibration is to be longer than 10 min. These permeabilities are an order of magnitude less than the permeabilities of the cellular membrane for these ions. Otherwise, the postulated concentration difference for the alkali metal ions could be maintained only under one of the following conditions. (a) If there were a shunt for Na^+, and possibly K^+, between the nuclear space and the extracellular medium as has been demonstrated for liver cells[24]. (b) If there were a pump in the nuclear membrane, which actively transported Na^+ into and K^+ out of the nucleus. Considering the first possibility, if the intranuclear Na^+ and K^+ concentrations are the same as the extracellular concentrations for these two ions, the cytoplasmic Na^+ concentration should be higher, not lower, than the overall cellular concentration, and the cytoplasmic K^+ concentration should be higher than the overall cellular K^+ concentration in those experiments in which both gradients are inverted. Since the AIB uptake is still active in such experiments (Table III, Part B) the driving forces opposing this transport would be greater than estimated above. Therefore, this interpretation can be dismissed.

Considering the alternative hypothesis, if the Na^+ and K^+ concentrations inside the nucleus are fixed owing to impermeability of the nuclear membrane, the concentration of Na^+ would have to be rather high in order to explain the data in terms of a cytoplasmic Na^+ concentration lower than the overall cellular Na^+ concentration. Inspection of the individual experimental samples shows that with the electrochemical potential gradients of both Na^+ and K^+ inverted, active AIB uptake can be observed even if the intracellular Na^+ concentrations are as high as 95 mequiv·l^{-1}. On the other hand, if the apparent impermeability of the nuclear membrane is due to an active ion pump, we would have a plausible explanation for all the data in terms of the ion gradients. In other words, the effective driving forces deriving from the electrolyte gradients would in all cases be in favor of active AIB uptake even if the apparent

gradients, *i.e.* those computed from the overall cellular Na^+ and K^+ concentrations, appear to be opposed to this transport. No evidence, however, has been found for such a pump in the nuclear membrane. Experimental investigations of the intranuclear Na^+ and K^+ concentrations under various conditions, especially during inversion of the overal ion gradients, should be carried out before this question can be answered definitively.

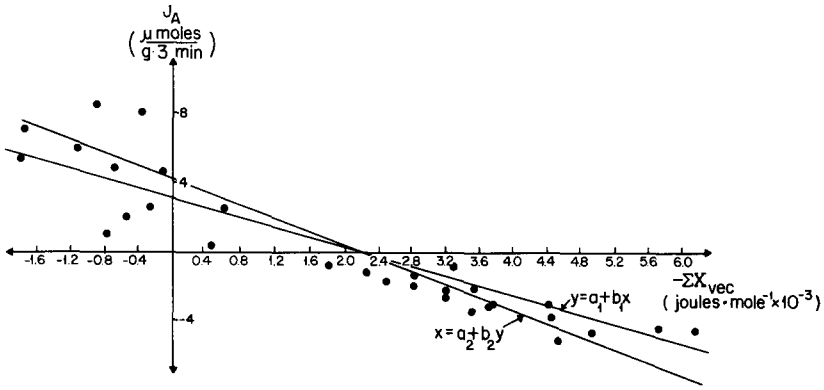

Fig. 3. The net influx of AIB as a function of the sum of the vectorial driving forces in the presence of 0.5 mM ouabain. The fluxes and forces are identical to those described in Fig. 2. The lines represent the least squares regression of y on x ($y = a_1 + b_1x$, $r = 0.91$) and of x on y ($x = a_2 + b_2y$, $r = 0.91$).

We have also attacked this problem in another way. Since the low Na^+ region should disappear after the Na^+ pump has been poisoned, experiments have been conducted in the presence of 0.5 mM ouabain (Fig. 3). As in Fig. 2, there are again well-correlated regression lines which do not go through the origin, and therefore, demonstrate that, even with adverse electrochemical potential gradients of all three solutes, active accumulation may occur. It is noteworthy, however, that the slopes of the regression lines are lower and that their X-intercepts are shifted considerably toward the left as compared to the results without ouabain (X-intercept, 2240 ± 192 joules·mole^{-1}). This effect may mean either that ouabain inhibits the amino acid transport directly, or that it inhibits the Na^+ pump incompletely, so that the Na^+ depleted region does not disappear entirely. In the latter case, the ouabain results do not exclude the possibility that the amino acid active transport is exclusively coupled to the electrochemical potential gradients of the alkali metal ions. More experiments are necessary to examine this possibility.

Any additional uptake of AIB *via* non-energetic exchange with endogenous amino acids can clearly be excluded as an alternative explanation of the deficiency in the vectorial driving forces. The pretreatment of the cells with AIB should have minimized the level of any endogenous amino acid or other solute capable of exchange with AIB. Furthermore, it has been shown by experiments with the autoanalyzer that none of the intracellular amino acid concentrations are appreciably changed during incubation with AIB (*cf.* Table II).

The remaining possibility, that amino acid active transport is coupled to cellular metabolism, is also supported by the work of POTASHNER AND JOHNSTONE[25] which shows that the rate of methionine uptake in Ehrlich cells is dependent on cellular ATP

levels for cells in both Na^+-replete and Na^+-free media. However, these authors feel there is no dependence of this uptake on the Na^+ gradient, but rather an absolute requirement for extracellular sodium. This conclusion is in direct contradiction to the results presented above (*cf.* Figs. 2 and 3) and to those reported by EDDY[14]. The discrepancy may result from the fact that POTASHNER AND JOHNSTONE did not consider the possible contribution of the K^+ electrochemical potential gradient which we have shown to be quite significant (*cf.* Table IV), and they report no K^+ concentration gradients from which this might be computed.

However, in our experiments which involve K^+ replacement of Na^+ in the bathing medium, we may have cause to question the effect of this ion shift directly on cellular metabolism. Indeed, HEALD[26] has shown that K^+ levels as low as 90 mM in the extracellular medium increased respiration by over 100 % of controls. Furthermore, ABADOM AND SCHOLEFIELD[27] observed that high extracellular K^+ concentrations greatly decreased glycine uptake by rat brain cortex. These investigators attributed this effect to the reduction in ATP levels, which they observed in the K^+-substituted media. Therefore, in such experiments reported here, the actual contribution of cellular metabolism to the uptake process may be diminished due to the effect of the high extracellular K^+ concentrations. If this were indeed the case, we might attribute an even greater fraction of the necessary energy expenditure under normal circumstances to cellular metabolism.

In summary, we are left with a net deficit of at least 4000 joules·mole^{-1} (2240 joules·mole^{-1} for experiments with ouabain) if amino acid active transport is energetically driven only by the electrochemical potential gradients of Na^+ and K^+. Within the limits discussed above, this missing energy would be most easily explained by a coupling to cellular metabolism, but a definite statement has to await further experimentation.

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