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Na^+ AND K^+ ELECTROCHEMICAL POTENTIAL GRADIENTS AND THE TRANSPORT OF α-AMINOISOBUTYRIC ACID IN EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. The uptake of α-aminoisobutyric acid (AIB) was measured in Ehrlich ascites tumor cells which were treated by osmotic shock and cold incubation to elevate cell Na^+ and lower K^+. These cells were suspended in media in which Na^+ was partially replaced by choline or K^+, thereby reversing the Na^+ concentration gradient or both the Na^+ and K^+ concentration gradients.

2. There was an inward transport of AIB against its concentration gradient when the Na^+ concentration gradient was reversed and also when both the Na^+ and K^+ concentration gradients were reversed.

3. In other experiments, the steady-state AIB and ion distribution ratios were measured, and the energy expenditure needed to maintain the AIB concentration gradient was calculated and compared with the energy expenditure calculated to be available from the ion electrochemical potential gradients in two model carrier systems.

4. A model in which there was a 1:1 cotransport of Na^+ and AIB and no associated K^+ movement was found to be inadequate to explain the entire AIB accumulation.

5. A model in which the carrier operated electro-neutrally, returning one K^+ for each Na^+ and AIB brought in, comes closer to satisfying the data from the studies of the steady state. However, there are a few discrepancies and the hypothesis does require a 100% efficiency of coupling between the ion fluxes and the uptake of AIB to explain most of the data from the steady-state studies.

INTRODUCTION

As early as 1952 CHRISTENSEN and his co-workers reported that replacement of Na^+ by K^+ or choline in the extracellular medium resulted in an inhibition of the uptake of amino acids by Ehrlich ascites cells[1,2]. CHRISTENSEN AND RIGGS[1] found that cellular K^+ was decreased and cellular Na^+ was increased in Ehrlich ascites cells incubated with glycine for 1–4 h. CHRISTENSEN *et al.*[2] suggested that there might be

Abbreviation: AIB, α-aminoisobutyric acid.

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a "common step in the processes by which K^+ and the amino acids are transferred" or that the energy necessary for the transport of amino acids might be derived from the potential energy available in the K^+ gradient. Later, RIGGS *et al.*[3] altered intracellular ion concentrations by various means; upon finding decreased amino acid uptake associated with decreased intracellular K^+ or increased extracellular K^+ they suggested that K^+ was acting like a competing amino acid and that a counterflow of intracellular K^+ down its gradient was responsible for the uptake of the extracellular amino acid. However, they also pointed out that in these experiments the Na^+ concentration was altered in a direction opposite to that of the change in the K^+ concentration and they suggested, as an alternative possibility, that the active transport of amino acid might depend on the Na^+ gradient if there was a cotransport of Na^+ and amino acid on a two-site carrier. At that time RIGGS *et al.*[3] preferred the former hypothesis primarily because of their finding that pyridoxal, a stimulator of amino acid transport, produced a greater loss of K^+ than a gain in Na^+.

For Ehrlich ascites cells the choice between these alternatives appeared to be resolved by the results of three studies. HEINZ AND PATLAK[4] reported that the free energy in the K^+ electrochemical potential gradient was approximately the same as that in the glycine gradient in the near steady state. HEMPLING AND HARE[5], however, found that the rate of free energy expenditure necessary to maintain the glycine steady state exceeded that made available by movement of K^+ down its electrochemical potential gradient even for an assumed 100 % efficiency of coupling beween the two fluxes. KROMPHARDT *et al.*[6] found that if extracellular Na^+ and K^+ were varied independently there was little change in glycine uptake when K^+ was changed at constant Na^+ but that glycine uptake decreased significantly when extracellular Na^+ was decreased at fixed K^+ levels. Therefore, these results pointed to a primary role for Na^+ rather than K^+ in this transport system.

Shortly thereafter, VIDAVER[7] showed that the uptake of glycine by pigeon red cells depended on extracellular Na^+ and that the initial velocity of uptake of glycine depended on the square of the extracellular Na^+ concentration. He proposed that two Na^+ moved in on the carrier for each glycine molecule transported. In a series of experiments in which he used the hemolysis and restoration technique with pigeon red cells, VIDAVER[8–10] showed that for cells so prepared the glycine distribution was directly dependent on the Na^+ electrochemical potential gradient and that when this gradient was reversed an active efflux of intracellular glycine was obtained.

A similar development has occurred for the active transport of sugars by intestinal mucosa. RIKLIS AND QUASTEL[11] and CSAKY AND THALE[12] showed that Na^+ is required in the mucosal bathing medium for the active transport of sugars. CRANE[13] proposed that a carrier with both a sugar and a Na^+ binding site was involved and that the active transport of the sugar was driven by cotransport of the Na^+ down its electrochemical potential gradient. Such a role for Na^+ was questioned by CSAKY[14] who proposed that a less specific effect of Na^+ and K^+ on some intermediate step common to more than one carrier in the intestinal mucosa was involved. CRANE[15] has since proposed a more comprehensive theory. Basic to this theory is the two-site carrier. On the basis of kinetic data CRANE proposed that there is an allosteric effect of the ion site on the sugar site such that when Na^+ occupies the ion site the carrier has a high affinity for the sugar but that when K^+ occupies this site the affinity of the carrier for sugar is greatly reduced. Thus, according to CRANE, the active transport

of the sugar is dependent on the "inward, downhill Na^+ gradient" and the "outward, downhill K^+ gradient". We will call this the Na^+–K^+ gradient hypothesis although CRANE has emphasized the Na^+ influx and the asymmetry of carrier–sugar affinites between the high K^+ intracellular side and the low K^+ extracellular side and has paid little attention to the K^+ efflux which, it would appear, is a necessary component of this hypothesis. CRANE[16] has shown that when intestinal mucosa which has accumulated 6-deoxyglucose from a Na^+-containing medium is placed in a $Tris^+$ medium containing the same concentration of 6-deoxyglucose, thus reversing the Na^+ concentration gradient, 6-deoxyglucose moves out of the cells. However, in these experiments CRANE used dinitrocresol and anoxia to inhibit cellular metabolism so they cannot prove that the ion gradients are the sole energy source for the sugar movement.

The work on amino acid transport in intestine has also converged recently on a recognition of the need for extracellular Na^+ for amino acid transport[17–22], and CURRAN[22] has proposed that the energy for amino acid transport in intestine might be obtained entirely from the Na^+ concentration gradient.

Further evidence for the role of Na^+ in amino acid transport in Ehrlich ascites cells was presented by WHEELER *et al.*[23] and INUI AND CHRISTENSEN[24]. They found that the saturable component of the flux of α-aminoisobutyric acid (AIB) depended on the first power of the extracellular Na^+ concentration suggesting a 1 : 1 stoichiometry of amino acid and Na^+ on a two-site carrier. We[25] measured the stoichiometry directly and found that the increase in the influx of $^{22}Na^+$ which occurred during transport of AIB and the saturable component of the influx of AIB were equal over a wide range of concentrations of extracellular Na^+ and AIB. WHEELER AND CHRISTENSEN[26] also reported an increase in Na^+ uptake accompanying amino acid uptake in red blood cells but they reported ratios that varied widely.

There are a number of problems associated with the ion gradient hypothesis for solute transfer. The first concerns the linkage of movement of ion and solute and the stoichiometry of this linkage. There is now good evidence for the coupling of Na^+ movement and movement of some amino acids or sugars for Ehrlich ascites tumor cells, red blood cells, leukocytes[27], intestinal mucosa, skeletal muscle[28] and kidney[29]. However, another problem is to determine whether all or only a part of the solute flux is driven by the ion gradients. Obviously coupled transport of an ion and a solute implies that a gradient in one can drive movement of the other, but this does not tell us whether solute movement is also coupled to cellular metabolism in other ways. Thus the demonstration that an ion electrochemical potential gradient drives solute movements in cells in which metabolism has been inhibited is part of the proof of coupling of the two movements. It provides evidence that the ion electrochemical potential gradient is the sole driving mechanism for the solute movement only if the solute flux is the same as in normal cells with the same ion electrochemical potential gradients. EDDY *et al.*[30] and EDDY[31,32] have recently compared the Na^+, K^+ and glycine movements in cyanide-treated and untreated Ehrlich ascites cells. EDDY[32] found that cyanide-treated cells did not accumulate glycine as strongly as did respiring cells which had the same Na^+ distribution ratio. He concluded that the Na^+ gradient did not provide all the energy for glycine accumulation and suggested an added contribution from the K^+ distribution or from the membrane potential. He favored a contribution from the distribution of K^+ and offered the empirical relationship for the distribution ratio of glycine,

$$\frac{[Gly]_i}{[Gly]_e} = \frac{[Na^+]_o}{[Na^+]_i} \cdot \frac{(1 + \theta[K^+]_i)}{(1 + \theta[K^+]_e)}$$

with $\theta < 0.025$ for cyanide-treated cells and $0.02 < \theta < 0.05$ for respiring cells. He did not consider the possible contribution from the membrane potential. We would point out that the membrane potential makes an appreciable contribution to the Na^+ electrochemical potential gradient and that if one uses only the Na^+ concentration gradient in evaluating the Na^+ gradient hypothesis, this is equivalent to assuming that the Na^+ and amino acid cotransport is electroneutral but that if one includes a contribution from the membrane potential one in effect assumes the cotransport is electrogenic. No doubt both electroneutral and electrogenic models should be tested.

In experiments reported here we have examined the time-course of AIB uptake in Ehrlich cells which had reversed Na^+ concentration gradients and reversed Na^+ and K^+ concentration gradients. WHEELER AND CHRISTENSEN[33] have measured alanine uptake in rabbit reticulocytes in the presence of a reversed Na^+ gradient and, although they obtained variable results, in some of their experiments alanine was concentrated in the presence of a small reversed Na^+ gradient. We have also examined the steady-state AIB ratios for cells with different Na^+ and K^+ gradients and compared the findings with those predicted by two particular models of the Na^+ gradient and the Na^+–K^+ gradient hypotheses, one electrogenic and one electroneutral. The results appear to be incompatible with the Na^+ electrochemical potential gradient acting as the sole source of energy for the AIB transport and also show some inconsistencies with the Na^+–K^+ gradient hypothesis. However, the latter cannot be completely excluded on the basis of these experiments.

METHODS

Preparation of cell suspensions

The line of Ehrlich ascites used in this laboratory, its maintenance and the collection of the cells has been described previously[34]. For almost all the experiments reported here, cell suspensions were prepared in the following manner. Exceptions are noted in the section on results. The ascites was collected into heparinized Krebs–Ringer phosphate. This suspension was diluted with an equal volume of cold (0–5°) demineralized water, was filtered through cheesecloth and was allowed to stand in a cold room (0–5°) for 10 min. It was then centrifuged at 65 × *g* for 10 min, and the cells were resuspended in Krebs–Ringer phosphate and centrifuged. They were resuspended in Krebs–Ringer phosphate containing ouabain and allowed to sit in the cold room for 0.5–1 h. This suspension was centrifuged, and the pellet was resuspended in the desired test medium for an experiment.

Composition of salt solutions

The standard Krebs–Ringer phosphate solution used in preparing the cell suspensions was made up by adding 10 ml of a phosphate buffer solution to each 46.5 ml of a basic salt solution which contained 145 mM NaCl, 5.76 mK KCl, 1.03 mM $CaCl_2$, 1.44 mM $MgSO_4$ and 1.44 mM KH_2PO_4. In a few of the experiments on the steady-state concentration ratios, the $CaCl_2$ and $MgSO_4$ were omitted. Na^+ and K^+ concentrations were varied by adjusting the relative amounts of NaCl, KCl and

choline chloride used in making up the basic salt solution. The actual concentrations of Na^+ and K^+ obtained in any experiment were measured with use of the flame photometer. The phosphate buffer added to the basic solution was made up from stock solutions of 154 mM NaH_2PO_4 and 100 mM Na_2HPO_4 except when we wanted to markedly reduce the extracellular Na^+ in which case the corresponding potassium phosphates were used. The pH's were 6.95–7.05 for the time studies but were varied considerably for the steady-state studies. Except for a few experiments in which hypertonic KCl was used, the osmolarities of the solutions were 286 $\pm$ 4 mosM.

Ouabain was added to give final concentrations of 0.25 or 0.5 mM in order to reduce the Na^+ pump efflux in most of the experiments. Stock ouabain solutions containing 127.6 or 255.2 mg of ouabain in 7 ml of absolute ethanol were prepared. 1 ml of the appropriate stock was added to each 100 ml of buffered salt solution.

Biochemicals and labeled compounds

Unlabeled AIB and ouabain were obtained from Calbiochem as A-grade biochemicals.

Tritiated AIB (generally labeled) was obtained from Nuclear-Chicago Corp. with a specific activity of 131 mC/mmole. The ^{14}C-labeled AIB was from New England Nuclear and had a specific activity of 8.7 mC/mmole. Stock solutions containing 100 μC/ml were made up with isotonic NaCl or with distilled water. In the experiments only 0.05–0.3 ml of these stock solutions were needed for 100 ml of cell suspension.

Time studies with reversed ion gradients

In all experiments, 125-ml erlenmeyer flasks with side arms were used. The suspension of Ehrlich ascites cells was pipetted into the main chamber, and the appropriate solution of AIB was pipetted into the side arm. Tritiated AIB was used in all the time studies. After a preliminary incubation of 1 min at 37°, the flasks were all tipped and mixed simultaneously. At the end of each incubation period duplicate samples were removed and plunged into an ice bath for 30 sec. The flasks were then taken to the cold room, and samples were taken as quickly as possible. For each flask, a 1-ml sample was pipetted into 12 ml of isotonic choline chloride at 0–5° and centrifuged for analysis of pellet activity; a 2-ml sample was pipetted into a tared 75 mm $\times$ 10 mm test tube and centrifuged to obtain supernatant solution for analysis and to obtain wet and dry pellet weights; a 2-ml sample was pipetted into a 100 mm $\times$ 13 mm test tube and centrifuged to give a pellet and supernatant solution for analysis for Na^+ and K^+.

Extraction procedures and the correction for pellet extracellular space have been described[34]. The samples were counted with use of a Nuclear-Chicago Model No. 725 liquid scintillation counter which was set up for channels ratio counting. The method of calculation of intracellular amino acid has been described[35]. It gives intracellular concentration in the units mmoles per kg intracellular water.

The samples for Na^+ and K^+ analysis were handled by standard methods. The pellets were prepared for analysis with use of osmotic lysis with distilled water and dilution with Li_2SO_4 to a final concentration of 14.41 mM in Li_2SO_4. The samples were read and compared with known standards with use of an NIL flame photometer with an internal lithium standard. In a few experiments chloride was also measured on these extracts with use of an Aminco-Cotlove chloride titrator.

Steady-state experiments

Except for the patterns of incubation these experiments differed little from the previous. ^{14}C-Labeled AIB was used in most of them and a 2-ml sample for analysis for radioactivity was taken and diluted with 12 ml of cold Krebs–Ringer phosphate rather than choline chloride. Radioactivity was measured with use of a Nuclear-Chicago Model No. 6853 liquid scintillation counter equipped with an external standard.

Statistics

For the time studies, the recovery of added [^{3}H]AIB was 98.8 ± 0.3 (S.E.)%. Duplicate samples were used for all the time studies. The mean coefficient of variation for the AIB distribution ratios was 1.85%; for extracellular Na^+ and K^+ it was 0.8% and for intracellular Na^+ and K^+, 2.6%. Duplicates were not run in the steady-state studies, but our general experience with [^{14}C]AIB is very close to that with [^{3}H]AIB.

RESULTS AND DISCUSSION

Time studies

Table I gives the results of two experiments in which the Na^+ concentration gradients were reversed at the start of the experiments. All but approx. 35 mM of the extracellular Na^+ was replaced by choline. Intracellular Na^+ exceeded extracellular Na^+ for at least the first 10 min of Exp. 1 and for the entire period of Expt. 2, yet in

TABLE I

TIME STUDY OF AIB DISTRIBUTION RATIOS FOR CELLS WITH A REVERSED Na^+ GRADIENT

Cells were prepared by the cold-shock procedure described and then incubated with the AIB at 37° in a medium in which the Na^+ was partially replaced by choline. Ouabain was present at 0.25 mM throughout all procedures. Each value presented is the average of duplicate samples.

Expt. No.	*Incubation period (min)*	*Initial* $[AIB]_e$ *(mM)*	*AIB distribution ratio* (c_i/c_e)	$[Na^+]$ *(mM)*		$[K^+]$ *(mM)*	
				Intracellular	*Extracellular*	*Intracellular*	*Extracellular*
1	2	2.0	2.40	84.7	35.3	89.9	26.8
	5	2.0	3.38	74.0	36.2	100.6	26.8
	10	2.0	4.18	58.8	37.4	102.6	26.8
	30	2.0	5.53	31.7	40.2	124.8	26.8
2	2	10.5	1.02	86.2	34.6	73.0	30.0
	5	10.5	1.38	79.3	36.0	80.8	29.8
	10	10.5	1.75	69.3	36.2	83.4	28.5
	30	10.5	2.46	46.7	38.8	98.6	28.6

both AIB was accumulated to considerable concentration gradients. A graph of the time-course of changes in the distribution ratios for Na^+, K^+ and AIB in Expt. 1 is given in Fig. 1. In the experiments reported in Table II, the initial concentration gradients in Na^+ and K^+ were both reversed. These gradients ran down more rapidly than did those in the experiments reported in Table I, presumably either because the Na^+ pump was not completely inhibited and Na^+ extrusion from the cells by the Na^+

TABLE II

TIME STUDY OF DISTRIBUTION RATIOS FOR CELLS WITH BOTH Na^+ AND K^+ GRADIENTS REVERSED

Cells were prepared by the cold-shock procedure and then incubated with AIB at 37° in a medium in which the Na^+ was partially replaced by K^+. Ouabain was present at 0.5 mM except in Expt. 3 in which it was present at 0.25 mM. Each value presented is the average of duplicate samples.

Expt. No.	*Incubation (min)*	*Initial $[AIB]_e$ (mM)*	*AIB distribution ratio (c_e/c_i)*	*$[Na^+]$ (mM)*		*$[K^+]$ (mM)*		*Cl^- equilibrium potential (mV)*
				Intracellular	*Extracellular*	*Intracellular*	*Extracellular*	
3	2	10.0	1.00	56.9	49.0	93.6	109.8	
	5	10.0	1.48	51.4	47.6	103.4	110.7	
	10	10.0	1.85	37.9	50.7	138.1	107.5	
	30	10.0	2.66	19.0	53.7	143.4	102.4	
4	0	2.0	—	68.4	31.0	97.9	124.9	—
	2	2.0	1.60	63.0	31.9	99.3	122.8	−5.8
	5	2.0	1.76	50.2	31.9	99.7	120.9	−4.5
	10	2.0	1.94	39.8	32.6	108.4	121.0	−5.3
	15	2.0	2.30	35.5	33.5	119.3	122.6	−2.0
5*	0	2.0	—	95.4	33.3	90.6	149.8	—
	2	2.0	1.52	89.6	35.3	92.5	146.2	−2.4
	5	2.0	1.60	72.2	35.4	102.1	149.6	−6.9
	10	2.0	1.74	62.8	36.6	109.7	142.4	−1.0
	15	2.0	1.78	57.9	37.7	124.8	145.3	−0.3

* A hypertonic buffer containing 30 mM NaCl and 150 mM KCl was used; see text.

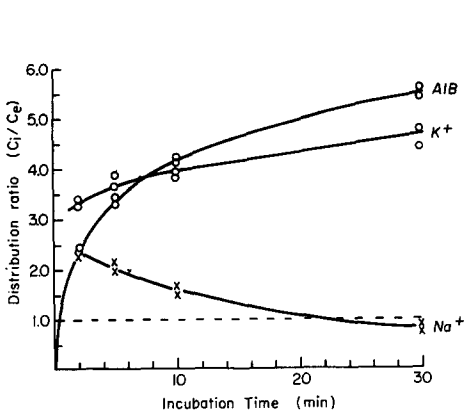

Fig. 1. Time-course of uptake of AIB in presence of a reversed Na^+ gradient. Expt. 1 in Table I.

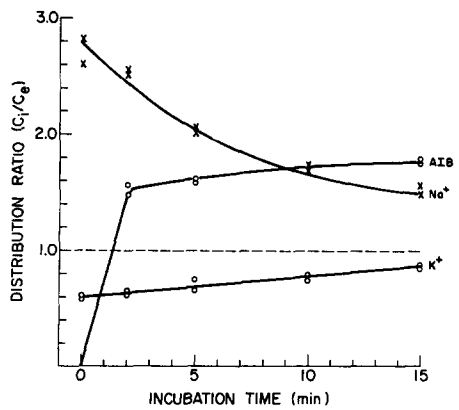

Fig. 2. Time-course of uptake of AIB in presence of reversed Na^+ and K^+ concentration gradients. Expt. 5 of Table II.

pump was increased by the increased extracellular K^+ or because extracellular K^+ exchanges with intracellular Na^+ more readily than does choline. In Expt. 3, both gradients were reversed for the first 5 min at which time the distribution ratio for AIB was 1.48. In Expts. 4 and 5, both ion concentration gradients were reversed for 15 min. For these two experiments the cells were first kept in the cold for 60 min, and the ouabain concentration was raised to 0.5 mM. In Expt. 5, we used a hypertonic KCl in making up the Krebs–Ringer phosphate in order to obtain a greater reversal

of the K^+ concentration gradient and to reduce the swelling which occurs in the higher concentrations of extracellular K^+ with isotonic solutions. The time-course of the distribution ratios for Na^+, K^+ and AIB in Expt. 5 is shown in Fig. 2. Distribution ratios greater than 1 for AIB were obtained with both ion gradients reversed, yet it should be noted that, at an extracellular concentration of 2 mM AIB, the distribution ratio for AIB was appreciably lower when both the Na^+ and the K^+ concentration gradients were reversed than when only the Na^+ concentration gradient was reversed. Cl^- was also measured in Expts. 4 and 5, and the calculated Cl^- equilibrium potentials are given in the last column of Table II.

From the results shown in Tables I and II, it appears that AIB is concentrated when the Na^+ concentration gradient is reversed and also when both the Na^+ and K^+ concentration gradients are reversed. However, it is also clear that AIB is not concentrated nearly as much when the Na^+ gradient is reversed as when the Na^+ gradient is "normal", *i.e.*, into the cell. This was to be expected since there is an impressive amount of evidence supporting the concept that Na^+ and AIB move into the cell on the same carrier and in a ratio of 1:1. Thus the fact that the concentration of AIB attained is further lowered when the K^+ concentration gradient is also reversed suggests some linkage to K^+ efflux as well. This is what one would expect if the return of the carrier, without AIB, from the inner to the outer surface of the cell membrane depended on the formation of a K^+–carrier complex at the inner surface of the cell membrane. This is one type of Na^+–K^+ gradient hypothesis. Although there is some evidence for an efflux of K^+ accompanying amino acid uptake in Ehrlich cells[1,5,30–32,36], there is no clear evidence as yet for a direct coupling between this efflux and the amino acid uptake. Thus the experiments shown in Tables I and II suggest that both the Na^+ and K^+ gradients are involved and yet that some concentration of AIB is obtained even when both concentration gradients are reversed. Hence in the experiments in which both Na^+ and K^+ concentration gradients were reversed there must be a coupling to another source of energy for the transport of AIB. At first it might appear that this conclusion eliminates the Na^+ and K^+ gradients as the sole source of energy for AIB transport in these cells and we so reported in a preliminary report[37]. However, the significant question is whether this extra energy is obtained *via* a direct linkage with cellular metabolism or from a coupling with another solute gradient. One possibility immediately comes to mind. OXENDER[38] has shown that Ehrlich ascites cells have appreciable levels of intracellular free amino acids which are approximately halved by the osmotic shock and washing procedure. Thus the initial flux of uptake of AIB could be in part an uptake in exchange for intracellular amino acids which is not dependent on the ion gradients. This consideration impelled us to seek a test of the Na^+ gradient and of the Na^+–K^+ gradient hypotheses which would necessarily have a lower contribution from exchange-driven counterflow. Studies at the steady state satisfy this requirement and indeed if endogenous amino acids are washed out by resuspending cells in fresh AIB solutions after an initial period of uptake, contributions from exchange diffusion should be minimized (Expts. 15–20 in Table III).

Steady state

We calculate the energy expenditure required to maintain the steady-state AIB concentration gradient and compare this with the maximum energy expenditure

TABLE III

SUMMARY OF RESULTS ON AIB DISTRIBUTION IN NEAR STEADY-STATES COMPARISON WITH PREDICTIONS OF MODELS 1 AND 2

Expt. No.	*Incubation time (min)*	*pH*	$[AIB]_e$ *(mM)*	$\frac{[Na^+]_e}{[Na^+]_i}$ *ratio*	$\frac{[K^+]_i}{[K^+]_e}$ *ratio*	*Model 2* $\frac{[AIB]_i}{[AIB]_e}$ *ratio*	*Model 2* $\frac{[Na^+]_e\,[K^+]_i}{[Na^+]_i\,[K^+]_e}$ *ratio*	*Model 1 (cal/mole)* $RT\ln\frac{[AIB]_i}{[AIB]_e}$	*Model 1 (cal/mole)* $RT\ln\frac{[Na^+]_e}{[Na^+]_i}$	*Membrane Potential needed (mV)*
8	30	7.0	About 1.0	3.23	16.22	23.8	52.3	1952	722	−53.5
1	30	7.0	2.0	1.32	4.66	5.53	6.16	1053	171	−38.3
2	30	7.0	10.5	0.83	3.45	2.46	2.86	554	−112	−29.0
6	75	7.0	1.8	0.86	4.03	4.22	3.46	887	− 91	−42.5
7	75	7.0	1.9	0.82	3.78	3.97	3.09	849	−123	−42.3
3	30	7.0	10.0	2.86	1.40	2.66	3.96	600	647	+ 2.0
9	50	6.34	1.91	1.07	5.78	3.03	6.17	685	42	−28.0
	50	6.75	1.81	1.12	6.10	4.00	6.86	855	70	−34.1
	50	7.06	1.81	1.14	6.92	4.38	7.87	910	81	−36.0
	50	7.33	1.85	0.87	7.34	3.29	6.41	735	− 86	−35.7
10	75	6.45	0.51	1.03	5.53	2.98	5.70	673	18	−28.5
	75	6.69	0.48	0.92	5.97	3.62	5.50	793	− 52	−36.7
	75	6.91	0.45	0.86	7.95	5.21	6.82	1019	− 91	−48.3
	75	7.10	0.47	0.75	8.48	4.84	6.37	972	−176	−49.9
11	60	6.38	0.62	1.19	1.10	1.66	1.30	312	107	− 8.9
	60	6.60	0.50	1.22	1.18	1.84	1.44	376	122	−11.0
	60	7.04	0.48	1.72	1.21	2.42	2.08	544	334	− 9.1
	60	7.32	0.53	1.44	1.25	2.30	1.80	513	225	−12.5

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12	60	5.60	0.43	0.91	1.11	3.44	1.01	761	− 59	−35.7
	60	6.60	0.50	1.42	1.24	1.85	1.76	379	216	− 7.1
	60	7.35	0.49	1.35	1.29	2.02	1.74	433	185	−10.8
13	62	7.08	0.66	1.52	1.22	2.24	1.85	497	258	−10.4
	62	7.08	1.18	1.15	1.28	2.22	1.47	491	86	−17.6
	62	7.08	2.16	1.31	1.25	1.92	1.63	402	166	−10.3
14	50	7.16	0.72	1.02	1.43	1.79	1.46	359	12	−15.1
	50	7.16	1.06	0.91	1.43	1.63	1.31	301	− 59	−15.7
15	65	7.17	0.47	1.92	1.34	3.06	2.58	689	402	−12.5
	65	7.14	0.73	2.58	1.32	2.83	3.41	641	584	− 2.5
16	65	7.15	0.50	4.58	2.87	9.17	13.14	1365	937	−18.6
	65	7.16	0.72	3.22	2.77	8.23	8.92	1299	720	−25.2
	65	7.18	1.36	2.97	2.68	7.71	7.97	1258	671	−25.5
17	65	7.12	0.82	1.50	13.86	5.75	20.79	1077	250	−36.0
	65	7.12	1.52	1.41	13.24	5.89	18.66	1092	212	−38.3
	65	7.12	1.93	1.37	11.97	5.73	16.38	1075	194	−38.8
18	65	6.00	0.66	1.49	1.35	4.32	2.01	901	246	−28.5
	65	6.00	1.43	1.47	1.37	2.60	2.02	589	237	−15.3
	65	6.00	1.89	1.42	1.32	2.16	1.88	474	216	−11.2
19	65	5.98	0.76	2.62	2.16	7.52	5.66	1243	593	−28.3
	65	5.98	1.48	2.31	2.22	5.39	5.14	1038	516	−22.7
20	65	5.97	0.81	1.20	13.32	4.58	15.96	937	112	−35.9
	65	5.97	1.53	1.43	9.61	3.57	13.72	784	220	−22.7

available from the ion electrochemical potential gradients for various models of coupling between the ion electrochemical potential gradients and AIB transport. For a model to be feasible, the rate of energy expenditure available from the ion electrochemical potential gradients must equal or exceed that required to maintain the steady-state AIB concentration gradient. First we derive a criterion of feasibility for a general model of the Na^+–K^+ gradient hypothesis and then look at two particular models.

Assume that the carrier binds k molecules of AIB and m of Na^+ at the outer surface of the cell membrane, then reorients in some way to the inner surface where it dissociates and then returns to the outside with n K^+ bound instead of the Na^+ and AIB. The net effect of one carrier cycle is to transfer k molecules of AIB from concentration c_e (outside) to concentration c_i (inside) and to transfer m Na^+ inward and n K^+ outward through their respective electrochemical potential changes across the cell membrane. In the steady state, the net carrier flux of AIB must just compensate for the rate of loss of AIB *via* all other routes. Let J_L be this flux; for brevity we will call it the leak flux of AIB, but this is used with no implication as to mechanism. Then the carrier-coupled fluxes of AIB, Na^+ and K^+ are J_L, mJ_L/k and $-nJ_L/k$, respectively. Note that there might be Na^+ and K^+ fluxes associated with other transports as well but that for this derivation we must consider only those coupled directly with the movement of AIB. The rate of energy expenditure required to maintain the AIB concentration gradient is given by Eqn. 1.

$$E_{AIB} = J_L RT \ln\left(\frac{c_i}{c_e}\right) = \left(\frac{J_L}{k}\right) kRT \ln\left(\frac{c_i}{c_e}\right) = \left(\frac{J_L}{k}\right) RT \ln\left(\frac{c_i}{c_e}\right)^k \tag{1}$$

The maximum energy obtainable from the coupled flux of Na^+, $m\,J_L/k$, is given by Eqn. 2 and that from the flux of K^+, $-n\,J_L/k$, is given by Eqn. 3.

$$E_{Na^+} = m\left(\frac{J_L}{k}\right)\left[RT \ln\left(\frac{[Na^+]_e}{[Na^+]_i}\right) - FV\right] \tag{2}$$

$$E_{K^+} = n\left(\frac{J_L}{k}\right)\left[RT \ln\left(\frac{[K^+]_i}{[K^+]_e}\right) + FV\right] \tag{3}$$

In Eqns. 2 and 3, F is the Faraday (23 cal·mole^{-1}·mV), V is the membrane potential in mV (outside taken as zero of potential) and subscripts i and e refer to intracellular and extracellular phases, respectively. The maximum rate of energy expenditure available from the Na^+ and K^+ electrochemical potential gradients by way of this mechanism is $E_{Na^+} + E_{K^+}$.

$$E_{Na^+} + E_{K^+} = \frac{J_L}{k}\left\{RT \ln\left[\left(\frac{[Na^+]_e}{[Na^+]_i}\right)^m \left(\frac{[K^+]_i}{[K^+]_e}\right)^n\right] - (m-n)FV\right\} \tag{4}$$

In order for the carrier-coupled ion movements to provide all the energy necessary for the maintenance of the AIB steady-state gradient, the rate of energy expenditure E_{AIB} must be less than or equal to $E_{Na^+} + E_{K^+}$. This gives us, as a criterion of feasibility,

$$RT \ln\left(\frac{c_i}{c_e}\right)^k \leq RT \ln\left\{\left(\frac{[Na^+]_e}{[Na^+]_i}\right)^m \left(\frac{[K^+]_i}{[K^+]_e}\right)^n\right\} - (m-n)FV \tag{5}$$

We wish here to consider two particular cases of this general model, an electrogenic model of AIB and Na^+ cotransport, and an electroneutral carrier cycle which is driven by the Na^+ and K^+ gradients. These models appear to be the most plausible in the light of current knowledge of the system.

Model 1: Electrogenic 1:1 cotransport of Na^+ and AIB. Assume that the carrier binds one Na^+ and one molecule of AIB at the outer surface of the cell membrane but that no K^+ are brought out; this is the case for which $k = 1$, $m = 1$, $n = 0$. The criterion of feasibility then reduces to Eqn. 6.

$$RT \ln \left(\frac{c_i}{c_e}\right) \leqq RT \ln \left(\frac{[Na^+]_e}{[Na^+]_i}\right) - FV \tag{6}$$

A similar model which assumes that one K^+ exits but no Na^+ enters on the carrier for each AIB entering could also be considered, but the evidence for the entry of one Na^+ for each AIB is so strong there seems little point to considering this.

Model 2: Electroneutral carrier cycle driven by Na^+ and K^+ gradients. Assume that, as in Model 1, one Na^+ and one AIB enter with the carrier but that one K^+ then attaches to the carrier at the inner surface and returns with it to the outer surface where it dissociates from the carrier. Thus the return of the carrier to the outer surface is driven by the K^+ electrochemical potential gradient. The net effect of a carrier cycle is to move one Na^+ and one AIB into the cell and one K^+ out, $k = m = n = 1$. Because the exchange of Na^+ for K^+ is 1 for 1, the contributions of the membrane potential in Eqn. 5 cancel out. Hence for this to be an energetically feasible mechanism,

$$RT \ln \left(\frac{c_i}{c_e}\right) \leqq RT \ln \left(\frac{[Na^+]_e}{[Na^+]_i} \cdot \frac{[K^+]_i}{[K^+]_e}\right) \tag{7}$$

which simplifies to the criterion

$$\frac{c_i}{c_e} \leqq \frac{[Na^+]_e}{[Na^+]_i} \cdot \frac{[K^+]_i}{[K^+]_e} \tag{8}$$

We see no point in considering other models at this time, in view of the stoichiometry of Na^+ and AIB movement on uptake[25,26]. However, it should be noted that a mixture of mechanisms can give rise to nonintegral values for k, m, n. For purposes of illustration, suppose that the fraction p of the AIB flux enters the cell by the mechanism given in Model 1 and the fraction $(1-p)$ enters by the mechanism given in Model 2. Then the rate of energy expenditure available from the ion movements coupled with the flux J_L of AIB is, the fraction p of Eqn. 4, evaluated for $k = m = n = 1$, plus the fraction $(1-p)$ of Eqn. 4, evaluated for $k = m = 1$, $n = 0$, which gives us Eqn. 9.

$$E_{Na^+} + E_{K^+} = J_L \left\{RT \ln \left[\frac{[Na^+]_e}{[Na^+]_i} \cdot \left(\frac{[K^+]_i}{[K^+]_e}\right)^{(1-p)}\right] - pFV\right\} \tag{9}$$

We are testing the plausibility of two extremes, Models 1 and 2; all possible mixtures of these two models give rates of energy expenditure between those of the two extremes.

Table III summarizes all experiments in which the cells were incubated for 30 min or longer. Ouabain was present in all experiments but Expt. 8. In Expts. 15–20,

the cells were incubated for 30 min with AIB at an extracellular concentration of 0.7–1.0 mM. The preparation was then chilled, centrifuged and resuspended in fresh AIB at the same concentration as before and incubated for another 15 min. This was done to wash away other amino acids which might have appeared in the extracellular phase as a result of exchange with endogenous stores and thus to reduce the levels of other amino acids present in these experiments. After the 15-min incubation, fresh Krebs–Ringer phosphate containing different concentrations of AIB was tipped in from a side arm in order to obtain extracellular concentrations of AIB which bracketed the original concentrations. The cells were then incubated for another 20 min, and the direction of change of intracellular concentration was followed. The last column in Table III gives the membrane potential which would be required if Model 1 were true. Consider the Na^+ gradient hypothesis first. Data on the membrane potential of Ehrlich ascites cells are not available for all of the different circumstances under which these cells were incubated. The most important experiment is Expt. 8. The results in Expt. 8 are typical of those obtained if no ouabain or metabolic inhibitor is present and the extracellular medium has the usual concentrations of Na^+ and K^+. For any model to be feasible, it must certainly explain the results obtained under these circumstances. Fortunately many measurements of membrane potential have been made on Ehrlich ascites cells under these conditions[39–41]. The recent studies[40,41] agree that the membrane potential in these cells is -11 to -13 mV. Thus the Na^+ gradient model does not even come near explaining the data of Expt. 8 unless one postulates some sort of compartmentalization of Na^+ in the cell so that the effective ratio, $[Na^+]_e/[Na^+]_i$, is about 15–16. Note that the Na^+–K^+ gradient model is adequate so far as the data in Expt. 8 are concerned. We also have a basis for estimating the membrane potential in Expts. 6 and 7. Cl^- was measured in these experiments, and if one assumes that the membrane potential is equal to the Cl^- equilibrium potential, we obtain a membrane potential of -28 mV, which is not enough to account for the AIB concentrations obtained in these experiments. However, HEMPLING AND KROMPHARDT[42] and AULL AND HEMPLING[43] have shown that only 60–70 % of the intracellular Cl^- is exchangeable. According to AULL[40] the membrane potential is a combination of the Cl^- and the Na^+ equilibrium potentials and replacement of extracellular Na^+ by a relatively nonpenetrating cation such as choline would increase the negativity of the membrane potential so that it would be approximately equal to that calculated on the assumption that 70 % of the intracellular Cl^- is exchangeable. Calculating the membrane potential on this basis, we obtain -37.5 mV for the cells in Expts. 6 and 7, still slightly short of the -42.5 mV needed. It seems clear that the particular Na^+ gradient hypothesis given by Model 1 is inadequate; it cannot explain the steady-state concentration ratios of AIB attained in all of these experiments.

The form of the Na^+–K^+ gradient hypothesis given by Model 2 is more difficult to eliminate on the basis of the data given in Table III. Clearly it comes closer to satisfying the energy requirements than does the Na^+ gradient hypothesis. In Fig. 3 are plotted all the data in Table III. For the Na^+–K^+ gradient hypothesis (Model 2) to be feasible energetically, all of the points should fall below or on the line $[AIB]_i/[AIB]_e = [Na^+]_e[K^+]_i/[Na^+]_i[K^+]_e$. Actually there are only three points that are without a doubt significantly above the line. Many of the points just above the line for ratios less than about 5 might be on the line when one considers all the

errors possible in these experiments, particularly the error in estimating the extracellular space of the pellet of cells. An upper bound for the error in the ratio $[AIB]_i/[AIB]_e$ is about 10% and the errors in the ratios $[Na^+]_e/[Na^+]_i$ and $[K^+]_i/[K^+]_e$ might be somewhat larger. Thus, taking the worst case, it is possible that in Fig. 3 a vertical deviation from the line of as much as 25% might be due to error. However,

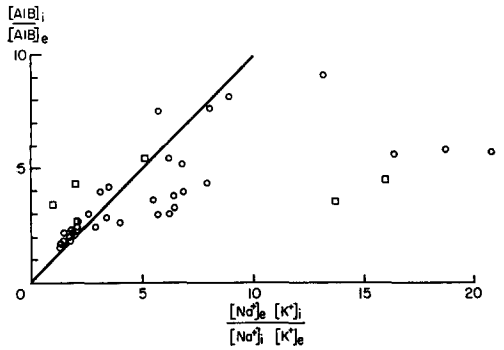

Fig. 3. Plot of near steady-state distribution ratio of AIB against $[Na^+]_e[K^+]_i/[Na^+]_i[K^+]_e$. Data of Table III; test of Model 2.

the three points highest above the line are certainly too far off to be explained in this way*. There is some small question about the two points at $[Na^+]_e[K^+]_i/[Na^+]_i[K^+]_e$ equal to 2.01 and 5.66 from Expts. 18 and 19, respectively, because the intracellular AIB was still falling, although very slowly, so that the ratio $[AIB]_i/[AIB]_e$ was still approaching the steady state from the high side. The same excuse cannot be made for the other high point, and we have no reason for believing that the data at this point are in error. Nonetheless, we would be reluctant to eliminate the Na^+–K^+ gradient hypothesis on the basis of these data alone, too much of it comes too close to satisfying the energy requirements. On the other hand, the latter conclusion does depend on an assumption of 100% efficiency of coupling between the ion fluxes and the AIB movement in Model 2. If this seems unreasonable and one admits the possibility of some energy loss in the coupling, then to that extent our data make Model 2 less likely an explanation as the sole source of energy for the uptake of AIB. Finally there are a number of other factors which should be considered, the effect of which would be to partly obscure relationships between the ion gradients and the AIB gradient. For one, the relations derived above should be in terms of activities, not concentrations. To see what effect this might have, consider Eqn. 8. It seems reasonable that if the activity coefficients do differ between intracellular and extracellular phases then the activity coefficient in the intracellular phase would be lower than in the extracellular. For the sake of the present argument assume this to be so. Let γ_{AIB}, γ_{Na^+} and γ_{K^+} be the ratios of activity coefficients in intracellular phase to those in the extracellular phase for AIB, Na^+ and K^+, respectively, all less than 1. We might expect γ_{Na^+} and γ_{K^+} to be nearly equal. If so then Eqn. 8 should really be

$$\gamma_{AIB}\frac{c_i}{c_e} \leqq \frac{[Na^+]_e}{[Na^+]_i}\cdot\frac{[K^+]_i}{[K^+]_e} \qquad (10)$$

* One of the referees has pointed out that the three points which are markedly high all come from experiments in which the pH was 6 or less, the $[AIB]_e$ was low and the $[Na^+]_e/[Na^+]_i$ was low.

Since $\gamma_{AIB} < 1$, this is a less stringent requirement than Eqn. 8. But we have no estimate of γ_{AIB}. Furthermore, any binding of AIB by intracellular large molecules would also contribute to an overestimation of c_i/c_e. Thus the effects of both of these factors would be in a direction which might well lead us to overestimate the left side of Eqn. 10. Whether they do so to a significant extent we do not know.

The coupling of Na^+ and solute fluxes demonstrated in many tissues and the less clearly established possible coupling with K^+ efflux in some tissues make the ion gradient hypotheses very attractive and simple mechanisms to explain the concentration of many solutes. Nonetheless we have to test whether the Na^+ and K^+ gradients can fully explain the concentration gradients of amino acids which are obtained. If Model 2 is a plausible explanation but Model 1 is not then some mixtures of Models 1 and 2 must also be plausible explanations. More definitive studies of the coupling of K^+ efflux with AIB influx are clearly needed.

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