J. Membrane Biol. 62, 149–160 (1981)

The Journal of
Membrane Biology

Amino Acid Active Transport and Stimulation by Substrates in the Absence of a Na^+ Electrochemical Potential Gradient

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Summary. Uptake of α-aminoisobutyric acid (AIB) was examined in Ehrlich ascites tumor cells treated with the cation-exchange ionophore nigericin (20 μg/ml). Membrane voltages were measured using the voltage-sensitive dye diethyloxadicarbocyanine (DOCC). In normal phosphate-buffered media, nigericin changed the distribution ratios of Na^+ and K^+ (the ratio of intra- to extracellular concentrations) nearly to unity, but AIB was still accumulated to a distribution ratio of ~9.0. When all but 40 mM Na^+ in the medium was replaced by choline, nigericin resulted in K^+ loss and Na^+ gain and both cation distribution ratios approached 2.8–3.4, as would be expected if both ions were distributing near electrochemical equilibrium with a membrane voltage in the range of −28 to −33 mV. This conclusion was supported by the observation that the addition of 5×10^{-7} M valinomycin to the nigericin-treated cell suspension produced no change in DOCC absorbance. In spite of the apparent zero electrochemical potential gradients for Na^+ and K^+, AIB was accumulated to a distribution ratio of 5.4 in the low-Na^+ medium. Addition of 0.1 mM oubain or 50 μM vanadate did not alter the extent of AIB accumulation as would have been expected if a large component of the membrane voltage were due to electrogenic operation of the $(Na^+ + K^+)$-ATPase. Addition of lactate, pyruvate or glucose increased the AIB distribution ratios to 11.9, 9.4 and 15.3, respectively. The effect of glucose could be explained, at least in part, by an enhanced Na^+ electrochemical potential gradient. However, neither lactate nor pyruvate produced any change either in membrane voltage or the intracellular Na^+ concentration. Therefore, these results confirm the existence of a metabolic energy source which is coupled to AIB accumulation and operates in addition to the Na^+ co-transport mechanism, and which is augmented by metabolic substrates such as lactate and pyruvate.

Key Words: Amino acid transport, Ehrlich ascites tumor cell, Na^+ co-transport, active transport, Na^+ electrochemical potential gradient, ATP, energy coupling, metabolism

It is widely accepted that a primary source for coupling energy to the active accumulation of amino acids in the Ehrlich ascites tumor cell and other cells is by co-transport of Na^+ down its electrochemical potential gradient. However, over the past decade several investigators have questioned whether this mechanism alone is sufficient to explain the steady-state amino acid accumulation, especially in the Ehrlich ascites tumor cell, which is capable of developing very high cell-to-medium distribution ratios of several amino acids. There are two reasons for such questions: the active accumulation of some amino acids in the absence of extracellular Na^+, and the apparent insufficiency of the Na^+ electrochemical potential gradient to explain steady-state distribution ratios.

Christensen, de Cespedes, Handlogten and Ronquist (1973) have shown that there is a class of amino acids, which is best characterized by the synthetic norbornane amino acid BCH, that is actively accumulated in the complete absence of extracellular Na^+. Prior depletion of endogenous intracellular amino acids and careful monitoring of amino acid loss from cells during BCH uptake show that exchange of extracellular BCH for intracellular endogenous amino acids cannot explain the active process (Christensen et al., 1973; Ohsawa, Kilberg, Kimmel & Christensen, 1980).

There are also studies which demonstrate that even Na^+-dependent amino acids such as α-aminoisobutyric acid (AIB) can be actively accumulated in Ehrlich ascites tumor cells when the transmembrane

0022-2631/81/0062-0149 $02.40

Na^+ concentration gradient has been reversed (Schafer & Jacquez, 1967; Jacquez & Schafer, 1969; Schafer & Heinz, 1971). However, it has not been determined whether the membrane voltage under these conditions was sufficiently negative (inside with respect to outside) to provide an inward-directed Na^+ electrochemical potential gradient, and questions have been raised about whether the Na^+ concentration gradient had been adequately assessed. When the Na^+ concentration gradient is reversed, this reversal is usually transient so that steady-state distributions of permeant ions cannot be used as indices of the membrane voltage. More recently there has been considerable success in using the carbocyanine dyes as voltage-sensitive probes which can respond rapidly to membrane voltage changes in Ehrlich cells (Laris, Pershadsingh & Johnstone, 1976; Philo & Eddy 1978*a, b*) and isolated membrane systems derived from these cells (Henius & Laris, 1979). Using this methodology, Philo and Eddy (1978*b*) concluded that methionine was accumulated by intact cells to a distribution ratio 1.8 times greater than the apparent Na^+ electrochemical gradient would predict. However, these investigators cautioned that the estimates of the Na^+ electrochemical potential gradient were based on intracellular Na^+ concentrations derived from total cell Na^+, and this calculated concentration might not be the same as that actually present at the cytoplasmic membrane surface. In fact, Pietrzyk and Heinz (1974) showed that Na^+ was accumulated in the nucleus in preference to the cytoplasm, which would favor a higher Na^+ electrochemical potential gradient than had been calculated in some circumstances. More recently, Johnstone (1978) has used the ionophore gramicidin to abolish transmembrane Na^+ concentration gradients and voltages in Ehrlich cells. It would be expected that this ionophore would also abolish intracellular compartmentation of Na^+. In the presence of a zero Na^+ electrochemical potential gradient, Johnstone (1978) found that the apparent K_m for glycine efflux was more than 10-fold greater than for influx, thus producing an energy-requiring flux asymmetry which could not be explained by the Na^+ electrochemical potential gradient.

As an alternative mode of energizing amino acid transport, Garcia-Sancho, Sanchez, Handlogten and Christensen (1977) have proposed that reducing equivalents (probably NADH) may be oxidized by a plasma membrane NADH dehydrogenase, which is coupled to amino acid transport. In support of this hypothesis, they showed that, when cells had been depleted of ATP by metabolic inhibitors, uptake of N-methyl-aminoisobutyric acid (N-methyl-AIB) could be restored by pyruvate or ascorbate plus phenazine methosulfate (PMS). In contrast, quinacrine (atebrin), an inhibitor of the NADH dehydrogenase, inhibited N-methyl-AIB uptake. The stimulation of uptake by substrates was observed to occur before substantial normalization of Na^+ and K^+ concentration gradients but not before cell ATP levels had also risen (Garcia-Sancho et al., 1977; Ohsawa et al., 1980). However, uptake was transient and the steady-state distribution of Na^+ compared to the amino acid could not be used as an argument against hyperpolarization of the membrane voltage as an explanation for the augmented uptake. In fact, Laris et al. (1976) and Pietrzyk, Geck and Heinz (1978) have shown that stimulation of the $(Na^+ + K^+)$-pump by high intracellular Na^+ produces substantial hyperpolarization of the membrane voltage. Such a hyperpolarization is a likely effect when amino acid uptake is restored in metabolically inhibited cells by substrate addition as in the experiments of Garcia-Sancho et al. (1977).

In the present study, we wished to examine the steady-state accumulation of AIB under conditions where the Na^+ electrochemical potential gradient was reduced to zero or near zero by nigericin, a cation-exchange ionophore, in much the same manner as Johnstone (1978) had used gramicidin for her kinetic studies. By using nigericin as a "clamp" on the Na^+ electrochemical potential difference, we were also able to study the effects of substrate addition in the steady state. Our results show substantial accumulation of AIB in the presence of a zero Na^+ electrochemical potential gradient. Even more striking, lactate and pyruvate produced dramatic increases in AIB accumulation without a change in either the membrane voltage or the Na^+ concentration gradient. Some of these results have also been reported in preliminary form (Jackson & Schafer, 1977).

Materials and Methods

The general methodology used for amino acid uptake studies in this laboratory has been described previously (Schafer & Heinz, 1971; Schafer, 1977). The tumor line, Ehrlich-Lettré (hyperdiploid), was provided three years ago through the courtesy of Dr. Charles Levinson (University of Texas Medical School, San Antonio, Texas) and has been maintained by regular transfer from mouse-to-mouse since that time. Tumor cells were harvested for experiments 7–10 days after injection and were not hemorrhagic by visual inspection.

Two basic solutions were used for most experiments. The first, referred to as normal-Na-KRP, contained (in mM): 130 NaCl, 10 Na_2HPO_4/NaH_2PO_4 (pH 7.4), 8 KCl, 2.0 $CaCl_2$ and 1.5 $MgSO_4$. In the other solution, referred to as low-Na-KRP, 100 mM of the NaCl was replaced by choline chloride and the sodium phosphates were replaced by potassium phosphates. In most experiments, these buffer solutions also contained 1% bovine serum albumin. However, in later experiments it was necessary to omit albumin from the bathing solution for absorbance measure-

ments using DOCC. In these cases, $CaCl_2$ was also replaced by an isosmotic equivalent of choline chloride. For all solutions the final osmolality was adjusted to 290 mOsm/liter and the final pH to 7.4.

AIB Uptake Experiments

After the cells were washed and resuspended in buffers as described previously (Schafer & Heinz, 1971; Schafer, 1977), 2 ml of suspension were placed in incubation flasks. The side-arms contained 2 ml of 2 mM ^{3}H-α-aminoisobutyric acid (AIB; New England Nuclear Corp., Boston, Mass.). Uptake was initiated by tipping to mix the two solutions, and was stopped in an ice-salt-water bath as described previously (Schafer, 1977). At the end of an incubation 100 μl of a solution of 290 mM sorbitol plus 0.5 μCi/ml ^{14}C-sorbitol (New England Nuclear Corp.) were added to each side-arm as the extracellular volume marker (Schafer, 1977). After centrifugation cell pellets were lyophilized to dryness and then extracted with 2 ml of deionized water for two hr. Aliquots of the extract were used to determine total pellet ^{3}H and ^{14}C dpm's by standard liquid scintillation techniques. Na^+ and K^+ in the extracts and supernatants were measured on a flame photometer, and Cl^- by electrometric chloride titration. Intracellular concentrations of ions and AIB were calculated from total pellet amounts, after correction for extracellular amounts, and intracellular water volume.

Measurement of DOCC Absorbance

Measurements of diethyloxadicarbocyanine iodide (DOCC) absorbance were made using an Aminco (American Instrument Co., Silver Springs, Md.) model DW-2 spectrophotometer in the dual-wavelength mode. Two ml of the cell suspension were placed in a 3-ml stirred cell, which was temperature-regulated at 37 °C in the instrument. DOCC was added to a final concentration of 3 μM and the absorbance was measured at 580 nM relative to 630 nM. The cytocrit was carefully maintained at 5%. After addition a rapidly decreasing absorbance difference was observed due to DOCC entry into the cells (Waggoner, Wang & Tolles, 1977; Rabon, Chang & Sachs, 1978). The signal reached a stable minimum within 5 min at which point valinomycin or substrates were added.

Determination of Intracellular ATP

In those experiments in which intracellular ATP concentrations were measured, the cell pellets were frozen and lyophilized immediately after centrifugation. They were then extracted with 3 ml of ice-cold 5% trichloroacetic acid for 1 hr at 0 °C. The resulting suspension was centrifuged at 0 °C and 0.25 ml of the supernate were neutralized with 2.25 ml of ice-cold 10 mM Na phosphate plus 4 mM $MgSO_4$ (pH 7.4). These extracts were kept at 0 °C for short periods or frozen when necessary, before they were analyzed for ATP according to the luciferase method of Stanley and Williams (1969).

Sources of Biochemicals

Nigericin sodium was kindly provided by Dr. R.J. Hosley of Lilly Research Laboratories (Indianapolis, Ind.). DOCC (Custom Synthesis, lot No. C3D) was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other biochemicals were obtained in purest available grade from Sigma Chemical Co. (St. Louis, Mo.).

Statistics

All experiments were conducted in a paired fashion so that control and experimental samples were derived from the same original cell suspension. Results are presented as mean values of several experiments with the standard deviation indicated. Observations of DOCC absorbance changes with substrates are shown in the form of a representative experiment. Each was repeated a minimum of three times with the same results.

Results

In this work we have examined the uptake of α-aminoisobutyric acid under a variety of conditions in which the Na^+ concentration gradient across the cell membrane was abolished or reversed. The electrochemical potential gradient for Na^+ was established by measuring the chemical gradient by standard flame photometry and the electrical gradient by absorbance changes of the voltage-sensitive dye, diethyloxadicarbocyanine iodide (DOCC). The Na^+ electrochemical potential gradient was manipulated by the use of the neutral cation-exchange ionophore nigericin and changes in the extracellular Na^+ concentration. The uptake of AIB was measured after addition of metabolic substrates or inhibitors such as ouabain.

Effect of Nigericin on Intracellular Composition and Cell Volume

In either the normal-Na-KRP or low-Na-KRP buffers, the cells maintained a constant low intracellular Na^+ concentration and a high intracellular K^+ concentration for at least 90 min. The addition of nigericin resulted in rapid loss of cell K^+ and a gain of Na^+. By examining the rate of change of intracellular K^+ and Na^+ with time of exposure to nigericin concentrations in the range 0.1–40 μg/ml ($0.2–7.2 \times 1.0^{-6}$ M based on a molecular weight estimate of 550 daltons), we determined that 20 μg/ml gave a maximal effect and this concentration was used in all subsequent studies.

Table 1. Effect of nigericin on intracellular cation concentrations and intracellular volume in normal-Na-KRP

Duration of incubation with nigericin (min)	$[Na^+]_i$ (mEquiv/liter)	$[K^+]_i$ (mEquiv/liter)	Intracell. H_2O g dry wt (g/g)
Control	22.0±1.0	152.5±2.5	2.84±0.03
11	140.6±8.9	20.6±2.2	3.38±0.01
20	150.5±9.2	17.0±1.2	3.49±0.10
40	147.8±9.1	16.2±1.2	3.57±0.14
70	152.7±7.3	15.6±1.0	3.83±0.08
100	151.3±8.3	14.9±1.2	4.04±0.40

Cells were incubated with 20 μg/ml nigericin at 37 °C for the times indicated. Control cells were incubated for 10 min at 37 °C in the absence of the agent. Measured extracellular concentrations were: $Na^+ = 153.1 \pm 1.8$ and $K^+ = 8.2 \pm 0.7$. Results are for five experiments.

Table 2. Effect of 20 μg/ml nigericin on intracellular ion and ATP concentrations, and cell volume, in low-Na-KRP

Duration of incubation with nigericin (min)	$[Na^+]_i$ (mEquiv/liter)	$[K^+]_i$ (mEquiv/liter)	$[Cl^-]_i$ (mEquiv/liter)	$[ATP]_i$ (mM)	Intracell. H_2O / g dry wt (g/g)
Control	17.4±3.6	177.6±7.1	98.6±11.4	2.3±0.2	2.92±0.10
11	80.5±4.8	95.4±2.2	79.2± 4.6	0.7±0.1	2.62±0.07
20	95.7±2.3	90.2±4.0	–	–	2.51±0.05
40	102.2±4.2	78.6±2.2	82.6± 8.4	0.5±0.1	2.46±0.06
70	114.2±4.2	79.5±1.7	–	–	2.40±0.07

Incubation times are for length of exposure to 20 μg/ml nigericin. All suspensions were incubated 10 min at 37 °C before nigericin was added. Measured extracellular ion concentrations were: $Na^+ = 41.6 \pm 1.1$; $K^+ = 23.7 \pm 1.0$; $Cl^- = 154.5 \pm 2.3$. Cation concentrations are averaged from 19 experiments, Cl^- concentrations from 4 experiments, and ATP concentrations from 10 experiments.

As shown in Table 1, in the normal buffer, addition of nigericin resulted in an almost equimolar loss of K^+ and gain of Na^+ as would be expected in the presence of the neutral exchange ionophore (Harris & Pressman, 1967; Henderson, McGivan & Chappell, 1969). Simultaneously, cell volume increased somewhat (Table 1, last column), but there was no evident change in membrane integrity: the ^{14}C-sorbitol space per g dry wt of cells increased from 1.02± 0.02 to a maximum of 1.19±0.06 at 70 min but there was no increase in the fraction of cells which stained with trypan blue.

As shown by Table 2, in the low-Na-KRP medium 20 μg/ml nigericin also produced rapid K^+ exit but in this case the Na^+ gain was only 80–90% of the K^+ loss, and the remaining fraction of K^+ loss was matched by an equal loss of Cl^-. There was, therefore, an anticipated and measured decrease in cell volume due to the relatively impermeant choline in the low-Na-KRP. As in the previous experiments, the ^{14}C-sorbitol space increased only slightly, from 0.95±0.05 ml/g dry wt to 1.13±0.04, and there was no increase in the fraction of cells which were stained by trypan blue.

Treatment with nigericin also resulted in lower intracellular ATP concentrations (Table 2) although the minimum ATP levels achieved were still greater than observed after treatment with metabolic inhibitors (Johnstone, 1974; Schafer, 1977). The reduction in cell ATP was probably due to at least two factors: increased load on the $(Na^+ + K^+)$-ATPase and the mitochondrial effects of nigericin (Henderson et al., 1969).

AIB Uptake in Nigericin-Treated Cells

The uptake of AIB from an extracellular concentration of 1.0 mM was examined in control cells and cells treated for 10 min with nigericin before addition of AIB. The results are shown in Figs. 1 and 2 for cells in normal and low-Na-KRP, respectively. For control cells in the normal Na-KRP, AIB reaches distribution ratios of *ca.* 23.0 within 30–60 min (Fig. 1). During the uptake, intracellular Na^+ and K^+ concentrations remained the same as shown for the control in Table 1. For the uptakes with nigericin, the cells were first incubated for 10 min at 37 °C with 20 μg/ml nigericin, then 1 mM AIB was added and uptakes were measured at the times after mixing as indicated in Fig. 1. There was marked AIB accumula-

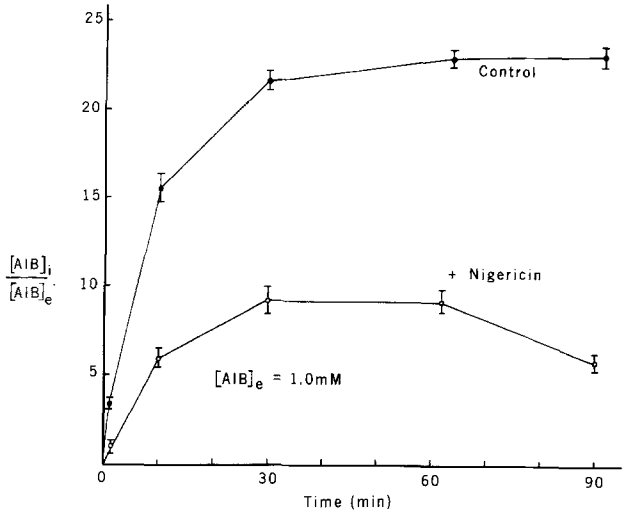

Fig. 1. Effect of nigericin on AIB uptake in normal-Na-KRP. The distribution ratio, intra- to extracellular AIB concentration ratio $[AIB]_i/[AIB]_e$, is plotted as a function of time of incubation with AIB. All cells were incubated at 37 °C for 10 min before AIB addition at zero time: control cells with no additions, experimental with 20 μg/ml nigericin present before and after AIB addition. The extracellular AIB concentration was 1 mM. Results of five experiments, in which control and nigericin observations were paired

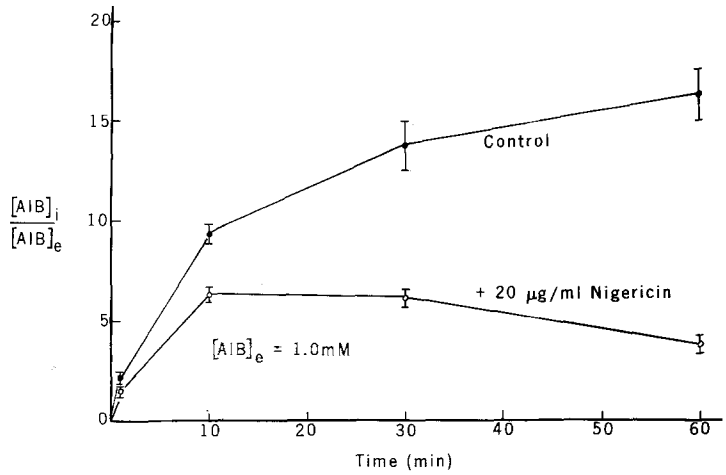

Fig. 2. Effect of nigericin on AIB uptake in low-Na-KRP. Experimental protocol was the same as described in the legend to Fig. 1. Results of four control experiments and nine nigericin experiments. The extracellular AIB concentration was 1 mM

tion in the nigericin-treated cells although less than in control cells as would be expected. During AIB uptake the Na^+ and K^+ concentration gradients followed the same time course as shown between 11 and 100 min in Table 1.

As shown in Fig. 2, in low-Na-KRP, uptake in control cells was less than in the normal medium as would be expected for the lower extracellular Na^+ concentration. During the uptake, intracellular Na^+ and K^+ concentrations remained the same as observed for controls (Table 2). In nigericin-treated cells, in which the Na^+ concentration gradient was strongly reversed, there was still significant active AIB uptake to a distribution ratio of 6.0. During AIB uptake intracellular Na^+ and K^+ concentration gradients followed the same time course as shown between 11 and 70 min in Table 2.

Transmembrane Voltages Determined by DOCC

The active uptake of AIB, observed in the presence of nigericin in low-Na-KRP medium when the Na^+ concentration gradient was reversed, could be explained by coupling to an inward-directed Na^+ electrochemical potential gradient only if the transmembrane voltage were significantly hyperpolarized under these conditions. In order to measure the membrane voltage we used the dye diethyloxadicarbocyanine iodide (DOCC). As reported by Laris et al. (1976) and Philo and Eddy (1978*a*) for the similar dye 3,3′-dipropyloxadicarbocyanine, addition of DOCC to a suspension of Ehrlich ascites tumor cells resulted in a decrease in DOCC fluorescence and in absorbance at 580 nM as would be expected for entry into the cells. Because carbocyanine dyes would also be expected to distribute into the mitochondria, we used metabolic inhibitors to abolish mitochondrial membrane potentials. Initially, we used the mixture of inhibitors suggested by Philo and Eddy (1978): 6 ng per ml (cell suspension) oligomycin, 1 μg per ml antimycin and 60 nmol per ml 2,4-dinitrophenol. This combination proved effective and we were able to reproduce the primary observations of Philo and Eddy (1978*a, b*). However, in the course of our work we found that another inhibitor combination, 0.1 mM tetraphenylphosphonium (TPP) plus 0.3 μM oligomycin was equally effective, and this combination was used in the studies reported here. Since the presence of albumin in the suspending medium gave artifactual changes in DOCC absorbance, it had to be omitted (Philo & Eddy, 1978*a*). The removal of albumin also necessitated omission of Ca^{++} from the medium to prevent cell clumping. Therefore, the evaluation of membrane voltages using DOCC necessitated several simultaneous changes in the protocol employed in the experiments reported above. Also, Smith, Herlihy and Robinson (1981) have recently reported that a similar carbocyanine dye inhibits O_2 consumption and intracellular ATP in Ehrlich ascites cells. Although the inhibition of oxidative phosphorylation was actually desirable in these experiments, it was possible that the dye could have other nonspecific effects. It was therefore important to examine the uptake of AIB under similar conditions, i.e. in the presence of 0.1 mM TPP, 0.3 μM oligomycin, 3 μM DOCC and 20 μg/ml nigericin, and with Ca^{++} and albumin omitted from the medium. The results are shown in Fig. 3 for five such experiments. In the presence of the new medium plus DOCC and the mitochondrial inhibitors, the peak AIB distribution ratio was about 20% lower than in the corresponding experiments shown in Fig. 2 (lower curve), and the fall in the distribution ratio after 10 min was more rapid. Nevertheless, the observed peak AIB distribution ratio was 4.8 and would require hyperpolarization of the membrane voltage to be explained on the basis of the Na^+ electrochemical potential gradient.

In order to measure the membrane voltage under these conditions, we used the null-point method, modified from that of Laris et al. (1976) and Philo and Eddy (1978*a*). Valinomycin was added at differing extracellular K^+ concentrations, and the change in DOCC absorbance was recorded and compared to the K^+ equilibrium voltage (E_K). The results of one such experiment are shown in Fig. 4. From the previous discussion, we would expect that in this particular experiment the actual membrane voltage lay between -20 and -44 mV. The combined results of five identical experiments are shown in Fig. 5. In this plot the intercept of each line with the X-axis is an estimate of the membrane voltage before valinomycin addition. For each experiment the X-intercept was estimated by a straight line connecting the data points for $E_K \simeq -27$ and -60 mV. X-intercepts were averaged for all experiments giving -32.4 ± 2.9 mV as an estimate of the membrane voltage. (Calculation of the intercept using polynomial fitting of the data points gave -30 ± 4 mV.)

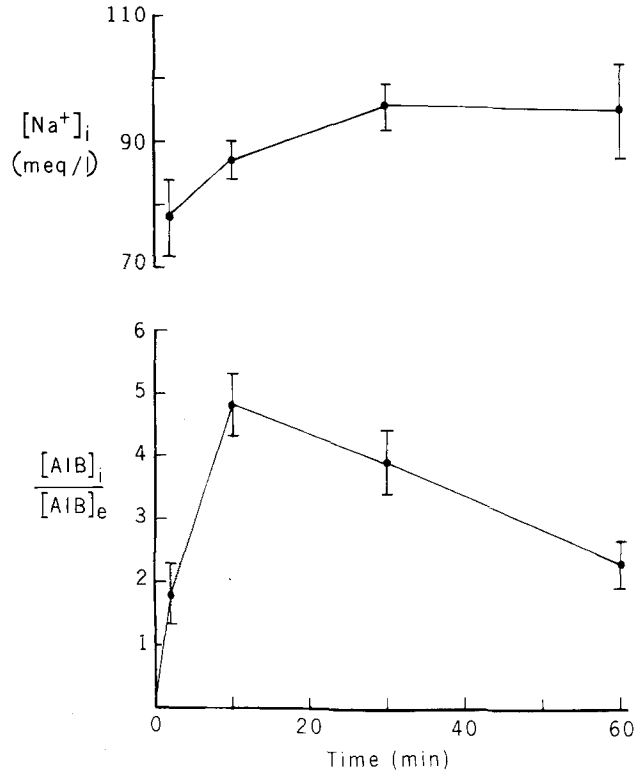

Fig. 3. Effect of mitochondrial inhibitors and DOCC on AIB uptake. The cells were incubated for 10 min at 37 °C in low-Na-KRP containing: 20 μg/ml nigericin, 0.1 mM TPP, 0.3 μM oligomycin and 3 μM DOCC, after which AIB was added at zero time. The upper plot shows the time course of intracellular Na^+, the lower plot is the time course of the AIB distribution ratio. Results of seven experiments

We attempted to perform the same null-point experiments in the presence of nigericin, using varying extracellular K^+ concentrations, but the nigericin caused a rapid collapse of the K^+ concentration differences so that we could not obtain a range of E_K values. However, some insight into the transmembrane voltage existing after nigericin treatment may be had by an examination of the predicted equilibrium (Nernst) potentials for the measured ions. From the intracellular concentrations presented in Table 2 for 40 min of nigericin treatment, the predicted equilibrium voltages (E, mV) would be: $E_{Cl} = -16.7$, $E_{Na} = -24.0$, and $E_K = -32.0$. The relative closeness of all three equilibrium voltages suggests that the actual membrane voltage may be in the range of -17 to -32 mV. Since, even after 40 min of incubation with nigericin, K^+ is still slowly leaving the cell and Na^+ is entering, approach to equilibrium with a membrane voltage of -20 to -30 mV would be a logical expectation. If our analysis is correct and the true membrane voltage in the presence of nigericin lies near to E_K, then addition of valinomycin after treatment with nigericin should produce no change in the DOCC distribution. These experiments were conduct-

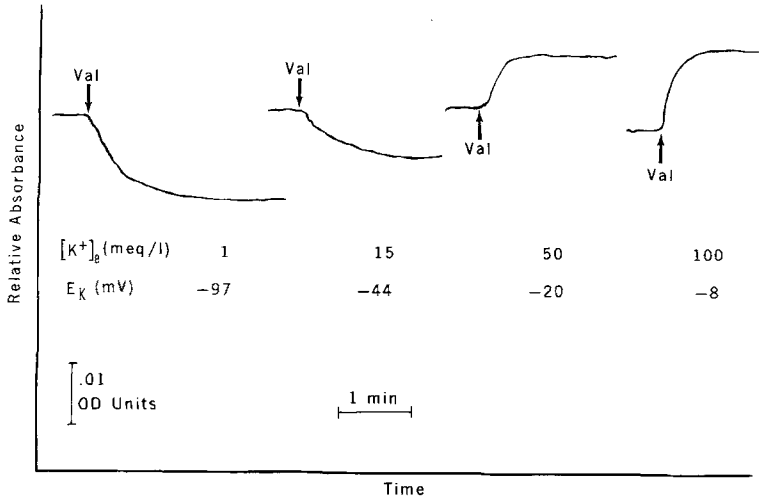

Fig. 4. Effect of valinomycin on DOCC differential absorbance with varying extracellular K^+ concentrations gradients. Cells were incubated in cuvettes at 37 °C in the indicated extracellular K^+ concentrations together with 3 μM DOCC and the metabolic inhibitors (*see text*). After a stable differential absorbance was obtained, 5×10^{-7} M valinomycin was added to the cuvette. A parallel sample was taken for measurement of intracellular K^+ from which the K^+ equilibrium voltage (E_K) was calculated

ed in the following way. Cells were incubated at 37 °C for 10 or 30 min with 20 μg/ml nigericin. DOCC was then added to the cuvette and the differential absorbance was followed until it reached a plateau, which occurred within 2 min. Then 5×10^{-7} M valinomycin was added and the change in differential absorbance was noted. After 10 min in nigericin, valinomycin produced an *increase* in the differential absorbance of 0.006–0.007 absorbance units, but there was no change in the differential absorbance with valinomycin after 30 min of incubation with nigericin, suggesting that the membrane voltage was near to E_K, i.e. approximately −30 mV. It was still possible although unlikely that in our DOCC experiments we were eliminating the proposed contribution of an electrogenic $(Na^+ + K^+)$-ATPase pump to the membrane voltage (Pietrzyk et al., 1978). If this electrogenic pump were responsible for a large fraction of the Na^+ electrochemical potential gradient, inhibiting the pump by ouabain should reduce the AIB accumulation. The results of four experiments designed to test this possibility are shown in Fig. 6. It can be seen that 10^{-4} M ouabain had no effect either on the Na^+ or the AIB distribution in nigericin-treated cells. In similar exper-

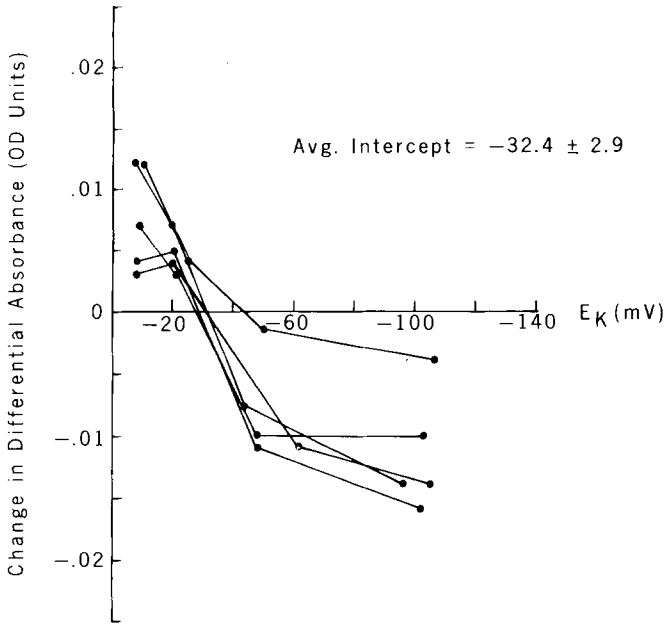

Fig. 5. Evaluation of membrane voltage, from changes in DOCC differential absorbance induced by valinomycin with varying K^+ concentration gradients. The changes in differential absorption with addition of valinomycin for five experiments such as that shown in Fig. 4 are plotted as a function of the K^+ equilibrium potential calculated from measured intra- and extracellular K^+ concentrations

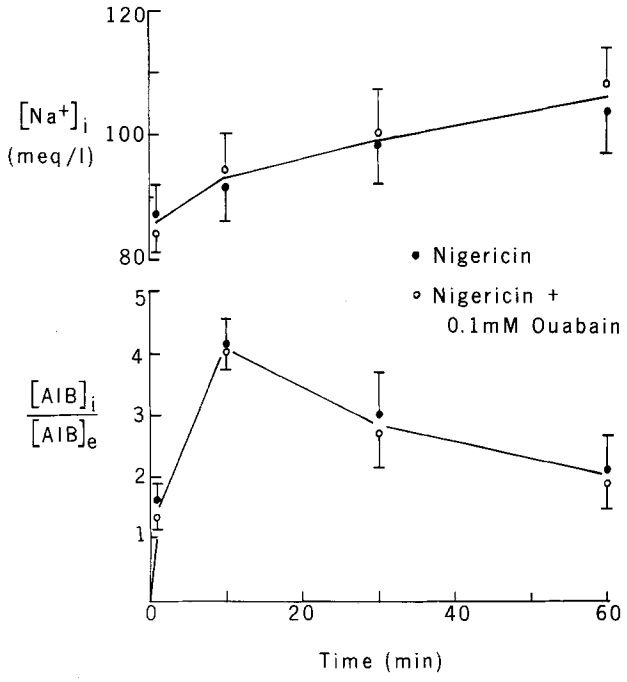

Fig. 6. Effect of 0.1 mM oubain on AIB uptake in the presence of nigericin. Cells were incubated for 10 min at 37 °C in low-Na-KRP either with 20 μg/ml nigericin or with 20 μg/ml nigericin plus 0.1 mM ouabain

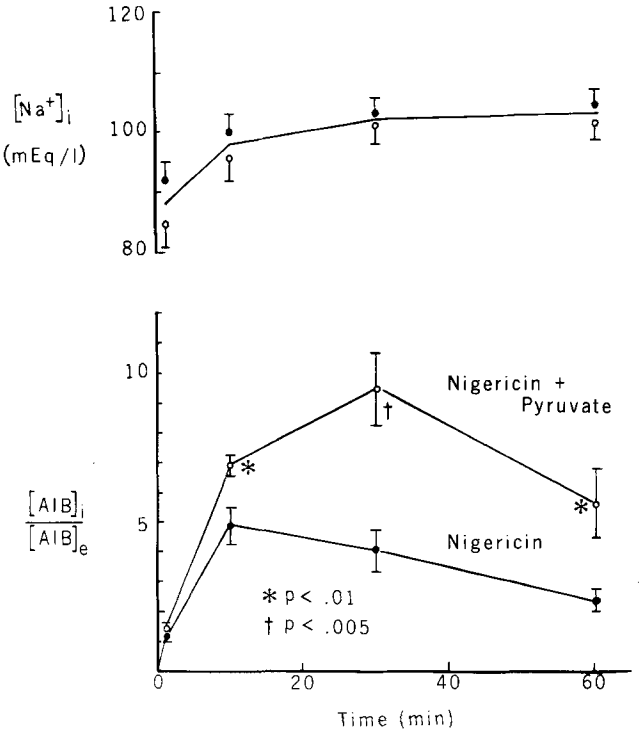

Fig. 7. Effect of pyruvate on AIB uptake and intracellular Na^+. Cells were incubated with 20 μg/ml nigericin in the presence or absence of 20 mM pyruvate for 30 min before adding 1.0 mM AIB. Results of five experiments. Measured extracellular Na^+ was 43.9 ± 1.3 mEquiv/liter

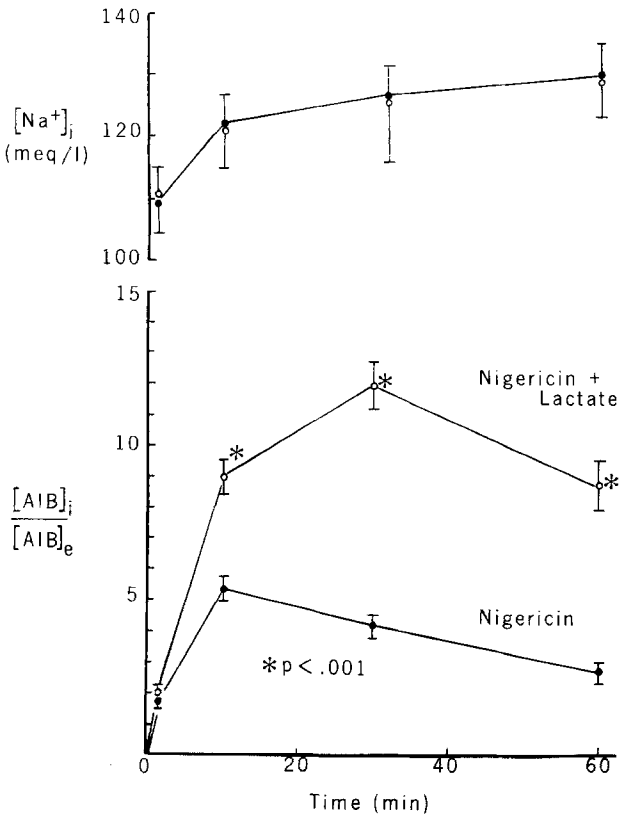

Fig. 8. Effect of lactate on AIB uptake and intracellular Na^+. Cells were incubated with 20 μg/ml nigericin in the presence or absence of 20 mM L-lactate for 30 min before adding 1.0 mM AIB. Results of six experiments. Measured extracellular Na^+ was 43.7 ± 0.5 mEquiv/liter

iments, which are not shown, we also found that 50 μM vanadate, a potent inhibitor of the $(Na^+ + K^+)$-ATPase (Nechay & Saunders, 1978), had no effect on either AIB or Na^+ distributions.

Effect of Metabolic Substrates on AIB Uptake in Nigericin-Treated Cells

In other experiments, we examined the effect of adding 20 mM pyruvate or L-lactate together with 20 μg/ml nigericin to the medium 30 min prior to adding AIB (Figs. 7 and 8). In both cases there was a dramatic increase in the uptake of AIB. In addition, the time course of AIB accumulation was prolonged. Instead of the peak accumulation occurring at 10 min after AIB addition, the peak accumulation was observed at 30 min and then declined after 60 min. In the case of lactate (Fig. 8), the AIB distribution ratio of 11.9 at 30 min, was nearly equal to the AIB distribution ratio of 14.0–16.0 achieved by cells incubated in low-Na-KRP without nigericin treatment (Fig. 2). In the latter experiments, however, the Na^+ concentration gradient was normal (~17 mEquiv/liter

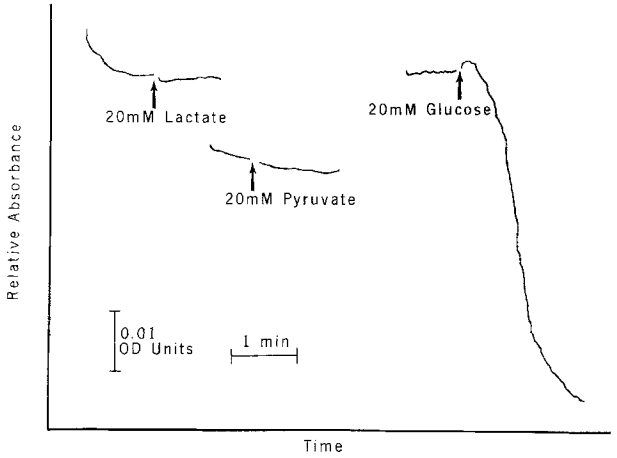

Fig. 9. Effect of lactate, pyruvate and glucose on DOCC differential absorbance. A decrease in relative absorbance indicates membrane hyperpolarization

intra- versus 42 mEquiv/liter extracellular), whereas in these experiments it was strongly reversed. Neither pyruvate nor lactate had any effect on the time course of the intracellular Na^+ concentration. Thus, if the effect of the substrates on AIB accumulation were produced by an increase in the Na^+ electrochemical potential gradient, this increase would have had to occur by the production of a more inside-negative membrane voltage. However, as shown in Fig. 9, DOCC differential absorbance was unaltered by the addition of lactate or pyruvate. In other experiments, 10–20 min incubation with lactate and pyruvate also had no effect on the differential absorbance of DOCC. In contrast, as we have shown previously (Heinz, Sachs & Schafer, 1981), addition of D-glucose to cell suspensions produced an immediate and dramatic decrease in differential absorbance due to DOCC (Fig. 9), which represents a marked hyperpolarization of the membrane voltage.

In view of the effect of glucose on the membrane voltage, we conducted experiments similar to those shown in Figs. 7 and 8 but using 10 mM glucose as shown in Fig. 10. Pretreatment with glucose resulted in marked stimulation of AIB uptake and the distribution ratios at 30 and 60 min were higher than those observed in low-Na-KRP in the absence of nigericin (Fig. 2). Furthermore, there was no tendency for the AIB distribution ratio to fall with time as seen in the case of lactate and pyruvate.

Finally, we examined the effect of lactate on intracellular ATP concentrations. As shown in Table 2, treatment with nigericin reduced intracellular ATP concentrations to less than 35% of control levels. However, after 10–30 min incubation with L-lactate in the presence of nigericin, the ATP concentration rose to 1.39 ± 0.20 mM (six experiments), i.e. about

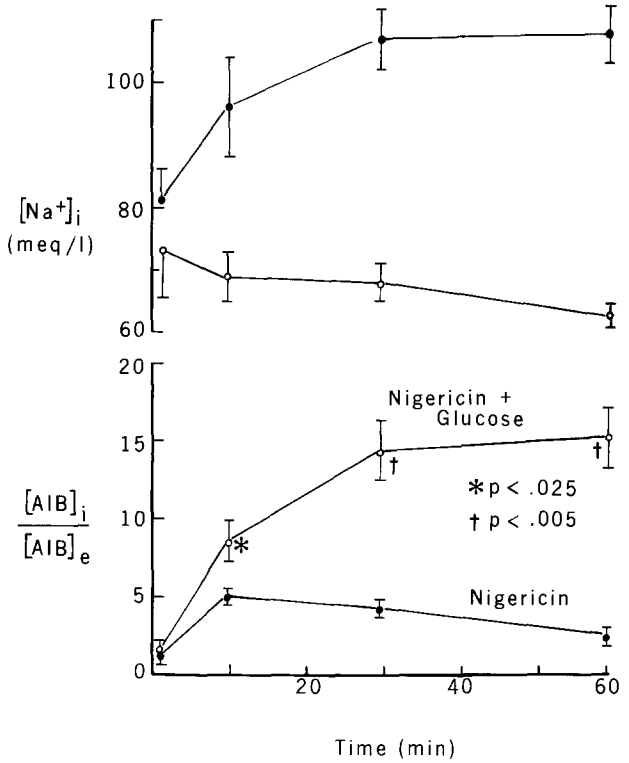

Fig. 10. Effect of glucose on AIB uptake and intracellular Na^+. Cells were incubated with 20 μg/ml nigericin in the presence or absence of 10 mM D-glucose for 10 min before adding 1.0 mM AIB. Results of four experiments. Measured extracellular Na^+ was 39.3 ± 1.2 mEquiv/liter

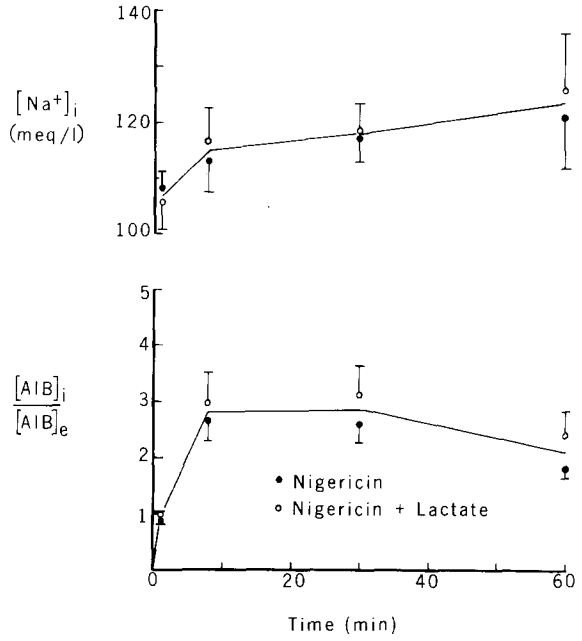

Fig. 11. Prevention of lactate stimulation by 2-deoxy-D-glucose. Cells were incubated with 20 μg/ml nigericin plus 10 mM 2-deoxy-D-glucose in the presence or absence of 20 mM L-lactate for 10 min before 1 mM AIB was added. Results of three experiments. Measured extracellular Na^+ was 43.2 ± 0.9 mEquiv/liter

two-thirds of controls level. Therefore, we wanted to see if prevention of the rise in intracellular ATP would affect the lactate stimulation of AIB uptake. In these experiments, the cells were preincubated either with 20 μg/ml nigericin and 10 mM 2-deoxy-D-glucose (DOG) alone or with 20 μg/ml nigericin together with 10 mM 2-deoxy-D-glucose plus 20 mM lactate. The DOG maintained the intracellular ATP concentration at less than 0.5 mM in all samples, and as can be seen in Fig. 11, it also prevented the stimulation of AIB uptake by lactate. In both groups of cells, uptake was reduced below that observed with nigericin treatment alone (Fig. 2).

Discussion

As would be expected from earlier work in red blood cells (Harris & Pressman, 1967), nigericin treatment of Ehrlich ascites tumor cells resulted in a rapid loss of cell K^+ in exchange for Na^+ influx. Although nigericin has a greater specificity for $K^+ - H^+$ exchange, it nevertheless has a significant Na^+ binding capacity and $Na^+ - K^+$ exchange would predominate at the relative concentrations of these three cations in normal media (Pressman, 1968). In the normal-Na-KRP medium, movement of the cations ceased within 20 min of nigericin addition (Table 1) with intracellular cation concentrations approximating those in the extracellular medium. We would assume from the rapidity of cation movement that both cations were at equilibrium with equal concentrations on each side of the cell membrane and a zero membrane voltage although this was not assessed directly in this medium. Due to the presence of the relatively impermanent cation choline, in the low-Na-KRP electroneutral exchange of intracellular K^+ for extracellular Na^+ did not produce a final equality of intra- and extracellular concentrations for these two cations. The loss of K^+ engendered by nigericin is matched by a nearly equal gain of Na^+ and the distribution ratios (inside to outside) for both cations approached 3.2. Since the exchange is expected to be electroneutral (Harris & Pressman, 1967; Henderson et al., 1969), the nearly equal distribution ratios do not necessarily mean that both Na^+ and K^+ are distributed at electrochemical equilibrium. However, the similarity of the reciprocal of the steady-state Cl^- distribution ratio and cation distribution ratios would suggest that all of the ions are nearly in electrochemical equilibrium with a membrane voltage in the range of -17 to -32 mV (inside

negative). Since all of the ions had nearly equal distribution ratios, a membrane voltage in this range would be anticipated if it were primarily a reflection of the diffusion potentials of these primary permeant ions. This conclusion would further suggest that electrogenic pump mechanisms are not a major determinant of the membrane voltage in this setting. Although Pietrzyk et al. (1978) have shown that an electrogenic ($Na^+ + K^+$)-ATPase pump mechanism can produce a considerable hyperpolarization of the membrane voltage in the presence of high intracellular Na^+, in the present setting this pump activity may be compromised by the reduced intracellular ATP levels occurring in the presence of nigericin (Table 2). Thus we observed no effect of either 0.1 mM ouabain or 50 μM vanadate on the AIB accumulation.

The conclusion that Na^+, K^+ and Cl^- are distributed near to equilibrium with a membrane voltage of about −30 mV is also supported by the absence of any change in differential absorbance of the voltage-sensitive dye DOCC when valinomycin is added after 30-min treatment with nigericin. This finding shows that the membrane voltage is near E_K, i.e. about −30 mV. In contrast, in untreated cells in low-Na-KRP with varying extracellular K^+ concentrations, addition of valinomycin produces the expected large changes in DOCC absorbance (Figs. 4 and 5). Also, in the nigericin-treated cells, glucose is able to produce a dramatic decrease in absorbance reflecting hyperpolarization (*see also* Heinz et al., 1981).

In previous attempts to define the magnitude of the Na^+ electrochemical potential difference ($\Delta\tilde{\mu}_{Na}$), not only have questions been raised regarding the assessment of the membrane voltage (e.g. Philo & Eddy, 1978*a*, *b*, Pietrzyk et al., 1978), but also about the possibility that measurements of intracellular Na^+ concentration from total cell Na^+ may not reflect the true cytoplasmic Na^+ concentration (Philo & Eddy, 1978*b*; Pietrzyk & Heinz, 1974). Due to possible sequestration in the nucleus (Pietrzyk & Heinz, 1974) or other unidentified intracellular compartments, the actual intracellular Na^+ concentration adjacent to the internal surface of the plasma membrane could be overestimated by measurements based on total cell Na^+, and hence $\Delta\tilde{\mu}_{Na}$ (outside with respect to inside) would be underestimated. In most of the present experiments, the extracellular Na^+ concentration was 40–43 mEquiv/liter, for which Pietrzyk and Heinz (1974) report no significant nuclear sequestration. Furthermore, the use of nigericin to "clamp" the plasma membrane $\Delta\tilde{\mu}_{Na}$ would also serve to dissipate any other Na^+ concentration gradients existing across intracellular membranes. Thus we would expect nigericin to abolish any intracellular Na^+ compartmentalization, at least if these compartments are bounded by lipid membranes. The observed shortfall in the Na^+ electrochemical potential gradient has also been observed during AIB accumulation in cytoplasts prepared from Ehrlich ascites tumor cells (Henius & Laris, 1979). These plasma membrane vesicles contain no interior compartments where Na^+ might be sequestered and the observed Na^+ electrochemical potential gradient was only 90% of that required to explain the steady-state AIB distribution (Henius & Laris, 1979).

From the above arguments, we expect that in both the normal and low-Na-KRP $\Delta\tilde{\mu}_{Na} \simeq 0$, yet there is continued, although transient, active AIB uptake. If one wished to argue that $\Delta\tilde{\mu}_{Na}$ is in fact greater than zero due to underestimates of membrane voltage in this setting, then the required voltage may be calculated from the approach of Jacquez and Schafer (1969). During the steady-state period when the AIB concentration gradient is maximal but not changing (*see* Figs. 1–3), the leak flux of AIB (J_{AIB}) must be matched by a co-transport of Na^+ (J_{Na}). The rate of dissipation of $\Delta\tilde{\mu}_{Na}$ under these circumstances must be at least as large as the energy conserved in maintaining the concentration gradient of AIB:

$$J_{AIB} \cdot \Delta\mu_{AIB} < n \cdot J_{Na} \cdot \Delta\tilde{\mu}_{Na} \tag{1}$$

where n is the stoichiometry of the coupled transport of Na^+ and AIB (i.e. the number of moles of Na^+ coupled to the flux of one mole of AIB).

After expressing $\Delta\mu_{AIB}$ and $\Delta\tilde{\mu}_{Na}$ in terms of the respective concentration gradients and the membrane voltage (V_m, mV), the required V_m is given by:

$$V_m < -\frac{RT}{F}\left(\frac{1}{n}\ln\frac{[AIB]_i}{[AIB]_e} + \ln\frac{[Na^+]_i}{[Na^+]_e}\right) \tag{2}$$

where R, T and F have their usual meanings. For the maximal AIB distribution ratio of 9.2 observed for nigericin-treated cells in normal-Na-KRP, the corresponding Na^+ distribution ratio was 1.0 (observed at the peak of AIB uptake in Fig. 1). Presuming a stoichiometry of 1.0, the membrane voltage would have to be at least as negative as −59 mV in order to explain the active AIB accumulation in terms of the Na^+ electrochemical potential gradient. Using the same analysis for AIB accumulation with nigericin-treated cells in the low-Na-KRP medium (Fig. 2), the peak AIB distribution ratio was 6.0 and the corresponding Na^+ distribution ratio was 2.3, giving the required voltage of −70 mV. It should be noted that the choice of higher stoichiometries (n) in Eq. (2) does not solve the problem. As n approaches infinity, the first term in brackets in Eq. (2) approaches 0 and the minimal required voltage becomes equivalent to the Na^+ Nernst potential. In short, the membrane voltages required exceed those measured, and the ar-

Table 3. Membrane voltage required for amino acid accumulation

Additions to bathing solutions	Incubation (min)	Peak uptake $\frac{[AIB]_i}{[AIB]_e}$	$\frac{[Na^+]_i}{[Na^+]_e}$	Required voltage (mV)
20 μg/ml nigericin (present in all protocols)	10–30	5.40±0.32	2.33	−68
+20 mM pyruvate	30	9.43±1.27	2.41	−83
+20 mM L-lactate	30	11.87±0.79	2.79	−93
+10 mM D-glucose	10	15.33±2.15	1.58	−85
+20 mM L-lactate and 10 mM DOG	30	3.11±0.57	2.73	−57

Results taken from experiments shown in Figs. 7–11. The membrane voltage required to explain the AIB accumulation on the basis of the Na^+ electrochemical potential gradient was calculated according to Eq. (2) (*see* Jacquez & Schafer, 1969) assuming an Na^+:AIB stoichiometry of 1.0.

guments presented above strongly suggest that $\Delta\tilde{\mu}_{Na}=0$. Voltages calculated according to Eq. (2) for a stoichiometry of 1.0 are summarized in Table 3.

The inadequacy of $\Delta\tilde{\mu}_{Na}$ in explaining AIB accumulation in this setting suggests the presence of an additional energy source which is coupled to amino acid accumulation. This conclusion is supported by the observed stimulation of AIB uptake by lactate and pyruvate (Figs. 7 and 8). In the case of lactate, the AIB distribution ratio approaches that seen in the absence of nigericin and with normal cation gradients (Fig. 2, upper curve). Since neither substrate changed the membrane voltage or the transmembrane Na^+ concentration gradient, the stimulation of AIB uptake occurred in the absence of any change in $\Delta\tilde{\mu}_{Na}$. Furthermore, both substrates are observed to prolong the time course of the AIB accumulation. Garcia-Sancho et al. (1977) observed similar effects of pyruvate and phenazinemethosulfate plus ascorbate addition to Ehrlich ascites cells which had been previously depleted of ATP by rotenone. The substrates were observed to restore N-methyl-AIB uptake before $\Delta\tilde{\mu}_{Na}$ had been brought back to normal. They interpreted their findings to suggest the involvement of energy from reducing equivalents which were oxidized at the plasma membrane, possibly with the participation of a plasma membrane NADH reductase (Garcia-Sancho et al., 1977). In support of this suggestion, these authors also observed that 1.8 mM quinacrine, an inhibitor of plasma membrane NADH reductase (Crane & Löw, 1976), prevented restoration of N-methyl-AIB uptake even when ATP levels and $\Delta\tilde{\mu}_{Na}$ were brought back to normal. Also, Ohsawa et al. (1980) found that NADH or PMS plus ascorbate added to the medium of inside-out vesicles from Ehrlich ascites cells enhanced N-methyl-AIB loss from the vesicles.

In experiments which are not shown here, we have also observed a small but significant stimulation of AIB uptake by 0.1 mM PMS+10 mM ascorbate under the same conditions as used for the pyruvate and lactate experiments (Figs. 7 and 8). We also found that 0.5 mM quinacrine inhibited the AIB uptake, such as observed in Fig. 2, by about 50%. However, this concentration of quinacrine also produced cell swelling and obvious deterioration of the cells as observed under the light microscope. With higher quinacrine concentrations, cells were almost completely disrupted. The observations with pyruvate, PMS plus ascorbate, and quinacrine support those of Garcia-Sancho et al. (1977) and Ohsawa et al. (1980); however, those with lactate (Fig. 8) do not. If the reduction of NADH were associated with energizing AIB uptake, we would expect opposite effects for pyruvate and lactate addition since they produce opposite changes in the NAD^+/NADH ratio in Ehrlich cells.

The stimulation of AIB uptake was, however, associated with an increase in cellular ATP levels. When the increase in ATP was prevented by addition of 2-deoxy-D-glucose, the lactate stimulation was also prevented. It has previously been suggested that ATP might be directly involved in energizing amino acid active transport. Johnstone (1974) showed that the initial rate of glycine uptake in Ehrlich cells fell in proportion to graded decreases in cell ATP produced by rotenone. Similarly, Ohsawa et al. (1980) have shown that in many settings intracellular ATP levels correlated well with amino acid distribution ratios when amino acid accumulation was stimulated in previously ATP-depleted cells by substrate addition. On the other hand, these investigators found no effect of ATP in stimulating amino acid exit from inside-out vesicles of Ehrlich cell plasma membranes (Ohsawa et al., 1980). Also, Geck, Heinz and Pfeiffer (1974) have shown on thermodynamic grounds that there is no coupling between AIB accumulation and ATP hydrolysis. Finally, it has been observed that Ehrlich cells can establish and maintain substantial distribution ratios of AIB after metabolic inhibition which virtually abolishes the cation gradients and lowers cell ATP to minimal levels (Schafer, 1977). Therefore, we think it unlikely that ATP is directly involved in energizing amino acid accumulation. However, cellular ATP levels may correlate with levels of some other high energy or redox substrate which is coupled to the amino acid transport system. The decrease in the amino acid distribution ratio with time in the presence of nigericin suggests that such energy supplies are limited but can be restored and maintained in the presence of pyruvate or lactate

(Figs. 7 and 8). We presently have no evidence as to the nature of this energy coupling, but we feel that its existence is established by these findings.

The authors thank Mrs. M. Crossman for secretarial assistance and Mrs. Ellen Bernstein for the illustrations. These studies were supported in part by an NIH Institutional Training Grant (5-T32-GM07195) award to Dr. Heinz and by NIH Research grants AM25519 and AM27060.

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Received 17 December 1980; revised 16 March 1981