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THE INFLUENCE OF CELLULAR AMINO ACIDS AND THE $Na^+ : K^+$ PUMP ON THE MEMBRANE POTENTIAL OF THE EHRLICH ASCITES TUMOR CELL

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Summary

The membrane potential of the Ehrlich ascites tumor cell was shown to be influenced by its amino acid content and the activity of the $Na^+ : K^+$ pump. The membrane potential (monitored by the fluorescent dye, 3,3′-dipropylthiodicarbocyanine iodide) varied with the size of the endogenous amino acid pool and with the concentration of accumulated 2-aminoisobutyrate. When cellular amino acid content was high, the cells were hyperpolarized; as the pool declined in size, the cells were depolarized. The hyperpolarization seen with cellular amino acid required cellular Na^+ but not cellular ATP. Na^+ efflux was more rapid from cells containing 2-aminoisobutyrate than from cells low in internal amino acids. These observations indicate that the hyperpolarization recorded in cells with high cellular amino acid content resulted from the electrogenic co-efflux of Na^+ and amino acids.

Cellular ATP levels were found to decline rapidly in the presence of the dye and hence the influence of the pump was seen only if glucose was added to the cells. When the cells contained normal Na^+ (approx. 30 mM), the $Na^+ : K^+$ pump was shown to have little effect on the membrane potential (the addition of ouabain had little effect on the potential). When cellular Na^+ was raised to 60 mM, the activity of the pump changed the membrane potential from the range −25 to −30 mV to −44 to −63 mV. This hyperpolarization required external K^+ and was inhibited by ouabain.

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Abbreviation: MOPS, morpholinopropane sulfonic acid.

Introduction

The Ehrlich ascites tumor cell is frequently used in studies of Na^+-dependent amino acid transport. In many of these investigations the activity of the Na^+ : K^+ pump and amino acid composition of the cell have been varied in order to design experiments to test the Na^+ gradient hypothesis. These manipulations, however, could alter the membrane potential, an important factor in the Na^+ gradient hypothesis, if the relation described in Eqn. 1 by Geck et al. [1] is correct * where P_K and P_{Na} are the permeability constants for ionic

$$E_m = \frac{RT}{F} \ln \frac{P_K[K^+]_o + (P_{Na} + P_C[S]_o)[Na^+]_o}{P_K[K^+]_i + (P_{Na} + P_C[S]_i)[Na^+]_i + A_{Na}} \quad (1)$$

diffusion for K^+ and Na^+; $[K^+]_o$, $[Na^+]_o$ and $[S]_o$ are the external concentrations of K^+, Na^+ and amino acid; $[K^+]_i$, $[Na^+]_i$ and $[S]_i$ are the internal concentrations of K^+, Na^+ and S; the term $P_C[S][Na^+]$ accounts for increased Na^+ movement with amino acids and A_{Na} represents the electrogenic movement of Na^+ through the Na^+ pump. It has not been demonstrated, however, that the cellular amino acid composition actually influences the membrane potential and the conditions under which the Na^+ : K^+ pump contributes to the membrane potential have not been clearly defined. The present study was planned to assess the influence of these two factors on the membrane potential using a fluorescent probe, 3,3'-dipropylthiodicarbocyanine iodide, to monitor the potential difference across the membrane [4].

The experiments reported below were also prompted by the previous report [4] of unexplained changes (an initial hyperpolarization followed by depolarization) in the membrane potential upon dilution of the cells. Since in theory either of these two factors (i.e., change in Na^+ : K^+ pump activity or changes in amino acid composition) could be responsible for the changes reported, experiments were performed to determine whether these factors played a role in the unexplained changes of the membrane potential.

While our results are only qualitative, they do indicate that both cellular amino acid levels and (Na^+ + K^+)-ATPase activity can significantly influence the membrane potential in these cells as predicted by Eqn. 1. Changes in membrane potential upon dilution of the cell suspension appear to be largely associated with losses of cellular amino acids. Preliminary accounts of portions of this work have already been presented [5,6].

Methods

The procedure for preparation of Ehrlich ascites tumor cells has been described previously [7]. After the cells were washed they were diluted and incubated as described in the text. Na^+-Ringer contained 154 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$ and 10 mM sodium phosphate buffer at pH 7.4. In K^+-free Na^+-Ringer, NaCl was substituted for KCl. K^+-Ringer and choline-Ringer were prepared by substituting equivalent concentrations of KCl or choline chloride

* This equation is a modification of the well known relationship formulated by Goldman [2] and Hodgkin and Katz [3].

for NaCl. In some experiments the buffer employed was 10 mM morpholinopropane sulfonic acid (MOPS).

The experimental procedures for Na^+, K^+, ATP determinations, and wet and dry weight measurements have been described previously [8,9]. The intracellular water compartment of the cellular pellets was determined from measurements of the dry weight, wet weight and [^{3}H]inulin distribution [4].

The fluorescent dye, 3,3'-dipropylthiodicarbocyanine iodide (referred to as the dye) employed in these studies was obtained from either Dr. Alan Waggoner of Amherst College or Dr. Stanley Parsons of the University of California at Santa Barbara. The fluorescent intensity of the dye in cell suspensions was measured as described previously [4]. The suspensions used in the fluorescence studies contained 3.3 mg dry weight per 10 ml, a dilution of 1 : 320 by volume. The final concentration of the dye in the cuvette was $3.0 \cdot 10^{-6}$ M. The temperature of the suspension in the Aminco-Bowman spectrophotofluorometer was regulated with a jacket through which water was circulated. In the text measurements of fluorescence are given in arbitrary units. Results from different experiments in most cases are not directly comparable for no effort was made to standardize instrument settings from day to day. Valinomycin and dye were added from stock solutions ($3.3 \cdot 10^{-4}$ M valinomycin and 0.5 mg/ml dye in ethanol). Ouabain was added as a dry powder.

Incubations were carried out in a gyratory water-bath shaker model G76 (New Brunswick, N.J.) using air as gas phase.

Analysis of amino acids in cells and fluid. Washed cells were diluted to 1 : 20 or to 1 : 320 with Na^+-Ringer and incubated at 37°C for 30 min. At zero time and 30 min aliquots were taken and centrifuged to yield pellets of approx. 0.5 ml cells. After the pellet was weighed, it was prepared for amino acid analysis using a modification of the method described by Stein and Moore [10]. The samples were then analyzed on a Beckman Model 120C Automatic Amino Acid Analyzer. Cell-free ascites fluid was also analyzed using the same method.

Amino acid uptake or flux. Washed cells were incubated with 2-aminoisobutyric acid (see text for concentrations) containing 2 μCi of 2-amino[1-^{14}C]-isobutyrate (New England Nuclear) per 100 ml cell suspension, for 30 min at 37°C at a 1 : 320 dilution in Na^+-Ringer. For uptake studies, duplicate 15-ml aliquots of the cell suspension were then centrifuged in tared tubes. After the supernatant fluid was removed, the tubes were wiped with tissue and the pellet was weighed. The pellets were then extracted with 0.5 ml 5% trichloroacetic acid. Aliquots (250 μl) of this extract were added to 10 ml of a mixture of Instagel (Packard) and scintillation grade toluene (Mallinckrodt) and analyzed for 2-amino[1-^{14}C]isobutyrate. In studies of amino acid loss, the cell suspensions were centrifuged after the 30 min incubation period and the supernatant fluid was removed. The cells were then diluted to 1 : 320 in Na^+-Ringer which did not contain amino acids. At intervals duplicate 15-ml aliquots were taken and analyzed for 2-amino[1-^{14}C]isobutyrate as described above.

$^{22}Na^+$ efflux. Washed cells were diluted 1 : 20 with Na^+-Ringer containing 2.5 μCi/ml $^{22}Na^+$ (New England Nuclear) and stored at 4°C for 1.5 h. The cells were washed once with cold choline-Ringer and three times with cold Na^+-Ringer. Aliquots of the cells were then resuspended in the test media. At intervals four samples of each cell suspension were taken and centrifuged. Aliquots of

the supernatant fluid were then taken for analysis. A sample of the whole cell suspension was also prepared for analysis by adding 100 μl of 50% trichloroacetic acid to 1 ml of cell suspension. After 30 min the cell sample was centrifuged and an aliquot of the supernatant fluid was taken for counting. Equivalent amounts of trichloroacetic acid were also added to the other samples taken. $^{22}Na^+$ was then counted on a Nuclear Chicago gamma counter. The fluxes were plotted using the equation described in Gardos et al. [11] for a two compartment system in the steady state.

Results

Internal amino acid pool

In our previous studies we reported that the membrane potential of cells diluted 1 : 320 (as they are in the cuvette in studies of fluorescence) originally appeared to be hyperpolarized (fluorescent intensity was low). Tests showed that initial hyperpolarization was not changed upon the addition of 1 mM ouabain to the cell suspensions. With time the cells underwent a gradual depolarization which was accomplished in 50—60 min at room temperature (ref. 4, Fig. 5) and more rapidly at 37°C (Fig. 1). The experiments described in Fig. 1 were done in two different ways. In one series of studies the cells were diluted to 1 : 320 with Na^+-Ringer and incubated at 37°C. Periodically an aliquot (3.2 ml) was taken, dye added, and the steady level of fluorescent intensity

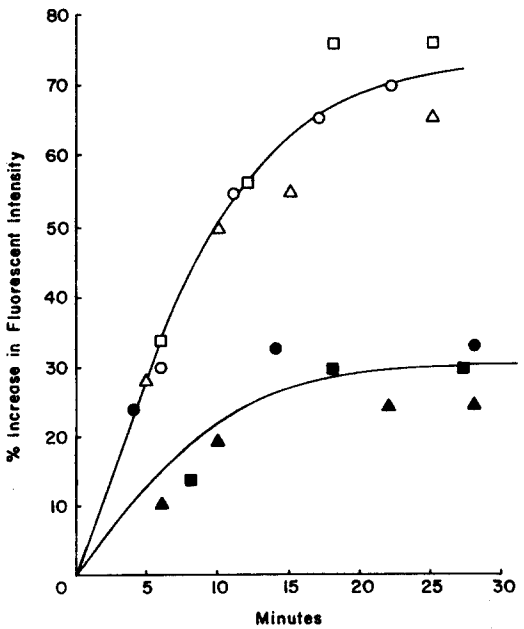


Fig. 1. Cells were diluted to 1 : 20 (closed symbols) or 1 : 320 (open symbols) in Na^+-Ringer and incubated at 37°C. At intervals 0.2 ml of the 1 : 20 cells were diluted with 3.0 ml Na^+-Ringer, dye was added and the steady level of fluorescent intensity recorded at 37°C. Similarly, 3.2-ml aliquots of the 1 : 320 dilution were taken, dye was added and the steady level of fluorescent intensity recorded. Results are plotted as the percentage increases in fluorescence based on the first sample recorded (three separate experiments are shown).

recorded. The data are expressed as the percentage increase in fluorescence based on the first sample taken. In a second series of experiments, the cells were diluted to 1 : 20 in Na^+-Ringer and then incubated at 37°C. At various times an aliquot (0.2 ml) was taken and diluted to 3.2 ml (1 : 320 dilution) at 37°C. Dye was then added and the steady level of fluorescence intensity recorded. In both sets of experiments an increase in fluorescence intensity was recorded indicating depolarization with time. The depolarization (as indicated by an increase in fluorescent intensity) was larger with the cells diluted initially to 1 : 320 than that with cells kept at 1 : 20.

Since Ehrlich cells have high endogenous levels of amino acids [12,13], we explored the possibility that the initial hyperpolarization upon dilution, followed by depolarization, was the result of a Na^+-coupled efflux of endogenous amino acids. (The possible involvement of the activity of the $Na^+ : K^+$ pump in this transient hyperpolarization followed by depolarization was eliminated by observations summarized later in the text.) According to this possibility the flux of Na^+ co-transported with endogenous amino acids would be high initially leading to hyperpolarization. It has been shown previously [14] that the efflux of amino acids is increased by intracellular Na^+ and we suggest that the large outward gradient for endogenous amino acids would result in an enhanced electrogenic Na^+ efflux through the co-transport mechanism. With time, as the internal amino acid pool is lost, the efflux of Na^+ would diminish and the cells would become depolarized. One would also predict that with greater dilution of the cells, the amino acid loss would be larger and hence the cells would show a larger final depolarization with time. To test this possibility the cellular amino acid pool was measured before and after a 30 min period of incubation at 37°C. The results show that the cells contain high concentrations of a number of amino acids (Table I) in line with earlier reports [12,13]. These amino acids are present in higher concentration in the cells than in the fluid of the abdominal cavity. When the cells are diluted 1 : 320 and incubated at 37°C, much of this pool is lost in 30 min. Substantial losses were also recorded when the cells were incubated at 1 : 20; these losses were smaller than those seen from cells diluted 1 : 320. The loss of substantial amounts of amino acids

TABLE I

CONCENTRATION OF VARIOUS AMINO ACIDS IN ASCITES FLUID AND IN CELLS BEFORE AND AFTER INCUBATION AT A DILUTION OF 1 : 320 IN Na^+-RINGER AT 37°C FOR 30 MIN

Amino acid	mmol/l water			Percent change in cells	
	Fluid	Cells		Here	Average (3)
		t = 0 min	t = 30 min		
Thr	0.166	2.35	0.65	−73	−77
Ser	0.120	1.50	0.50	−67	−64
Pro	0.210	3.89	0.49	−87	−94
Gly	0.274	6.50	1.70	−74	−74
Ala	0.529	8.68	0.52	−94	−90
Glu	0.183	8.64	2.21	−74	−60
Asp	0.071	2.65	9.16	+346	+550

during the time when the cells are depolarizing is consistent with the idea that the cellular amino acid concentration could be a factor in determining the membrane potential. Moreover, as predicted, greater cell dilution led to a greater loss of amino acids with an increased depolarization (Fig. 1).

When cells are incubated in media containing amino acids co-transported with Na^+, they depolarize as they accumulate amino acids and then repolarize as the steady-state level of accumulation is attained reaching approximately the same level of fluorescent intensity as cells incubated in amino acid-free media [4]. Hence the fluorescent intensity of cell suspensions incubated at 37°C at a 1 : 320 dilution in Na^+-Ringer in the presence and absence of 3 mM 2-aminoisobutyric acid show the same levels of fluorescent intensity after 30 min (Fig. 2). The cells which were incubated with 2-aminoisobutyrate in this manner now contain large quantities of 2-aminoisobutyrate and analyses show that they have lost as much of their endogenous amino acid pool as was lost from cells incubated without 2-aminoisobutyrate. The influence of cellular amino acids, in this case 2-aminoisobutyrate, on the membrane potential can

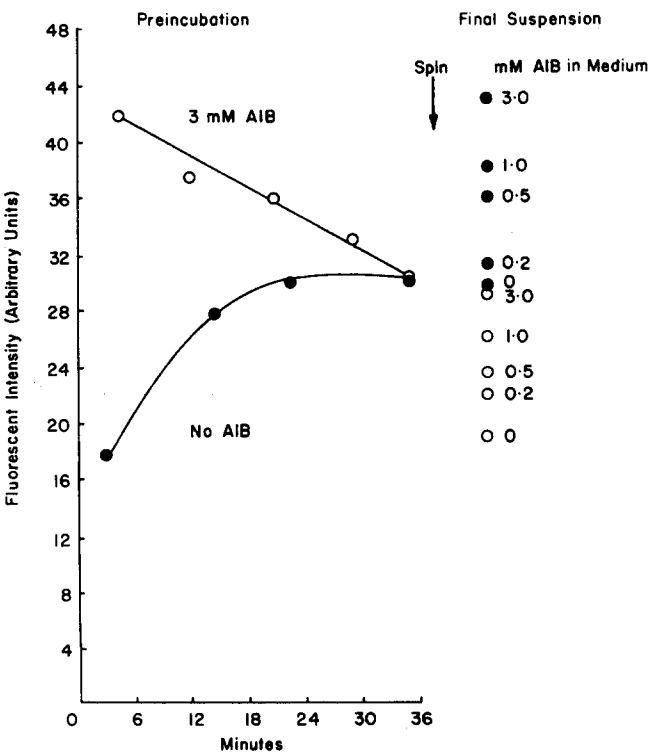


Fig. 2. Cells were diluted to 1 : 320 in Na^+-Ringer in the presence (open symbols) and absence (closed symbols) of 3 mM 2-aminoisobutyric acid (AIB) and incubated at 37°C. At intervals aliquots were taken; dye was added; and the steady level of fluorescent intensity recorded. After 36 min the cells were spun down and resuspended at a dilution of 1 : 20 ± 3 mM 2-aminoisobutyric acid (same as initial suspension). Aliquots (0.2 ml) of the suspension were taken and diluted to 1 : 320 with 3.0 ml of various concentrations of 2-aminobutyric acid (see graph) in Na^+-Ringer; dye was added; and the steady level of fluorescent intensity recorded. The position of the symbols underneath the medium 2-aminoisobutyric acid concentration indicates the fluorescence attained with the given concentration of 2-aminoisobutyric acid.

be shown when the fluorescent intensity of cells equilibrated with and without 2-aminoisobutyrate is compared after the cells are transferred to different media. In the following series of experiments the cells containing 2-aminoisobutyrate were spun down and resuspended at 1 : 20 and then diluted to 1 : 320 in Na^+-Ringer containing different concentrations of 2-aminoisobutyrate. If cells were resuspended in the same medium in which they were equilibrated, the potential did not change. Cells preincubated with 3 mM 2-aminoisobutyrate, however, hyperpolarize when placed in media containing less than 3 mM 2-aminoisobutyrate, the degree of hyperpolarization being greatest where the 2-aminoisobutyrate gradient is steepest. The hyperpolarizations were not changed in the presence of 1 mM ouabain. On the other hand, cells preincubated in amino acid-free media depolarize when exposed to 2-aminoisobutyrate, the degree of depolarization being greatest where the 2-aminoisobutyrate gradient is again steepest (Fig. 2).

The hyperpolarization recorded when cells that had accumulated 2-aminoisobutyrate were placed in Na^+-Ringer free of amino acids is only temporary. Immediately after dilution, the hyperpolarization is at its highest level. Thereafter the cells progressively depolarize (Fig. 3) toward the original pre-equilibrated level (see legend to Fig. 3 for methods). Parallel measurements of cellular 2-amino[1-^{14}C]isobutyrate concentrations with time were made on these cells, and as can be seen in Fig. 4, progressive depolarization can be correlated with a parallel loss of cellular 2-aminoisobutyrate (two separate experiments shown).

The influence of cellular amino acid concentration on the membrane potential was also seen in another series of experiments in which the magnitude of the transient hyperpolarization was shown to be dependent on the cellular 2-aminoisobutyric acid concentration. Cells were equilibrated with different

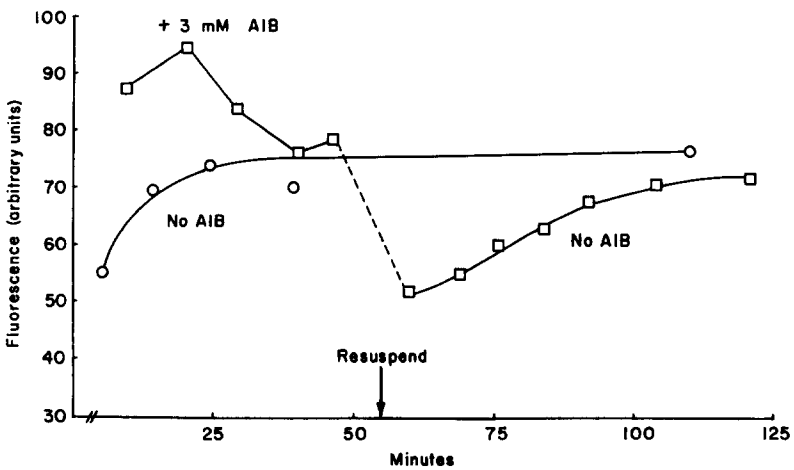


Fig. 3. Cells were diluted to 1 : 320 in Na^+-Ringer in the presence and absence of 3 mM 2-aminoisobutyric acid and incubated at 37°C. At intervals, dye was added to aliquots and the steady level of fluorescent intensity recorded. After 1 h the cells incubated with 2-aminoisobutyric acid were spun down and resuspended in Na^+-Ringer in the absence of 2-aminoisobutyric acid. After an initial hyperpolarization the trend is to depolarization.

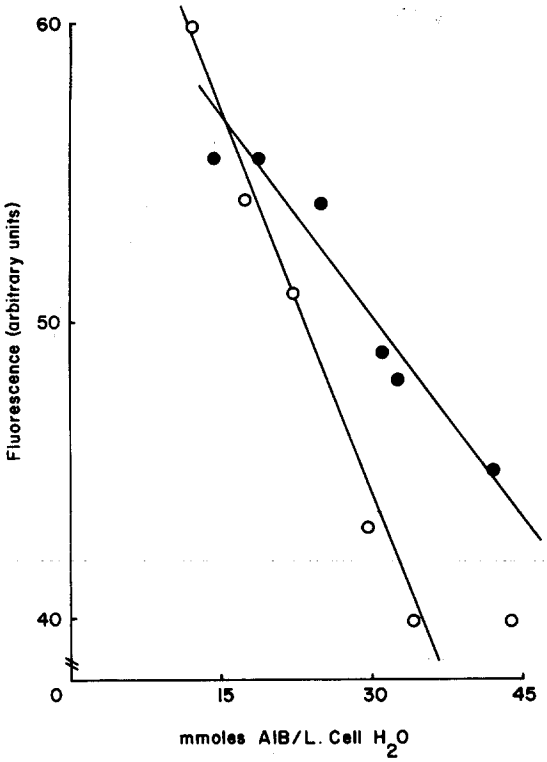


Fig. 4. Cells were diluted to 1 : 320 in Na^+-Ringer containing 3 mM 2-aminoisobutyric acid + 2-amino-[^{14}C]isobutyric acid and incubated at 37°C. After 35 min the cells were spun down and then resuspended in Na^+-Ringer in the absence of 2-aminoisobutyric acid. At intervals, dye was added to aliquots, and the steady level of fluorescent intensity recorded. Simultaneously, aliquots were taken and the cells were analyzed for 2-aminoisobutyric acid content.

concentrations of 2-aminoisobutyrate and then spun down and resuspended in media free of amino acids. As is seen in Fig. 5, in the initial period after resuspension in amino acid-free media the steady level of fluorescent intensity is lower with cells incubated in higher 2-aminoisobutyrate concentrations indicating that with higher cellular 2-aminoisobutyrate, there is greater subsequent hyperpolarization.

The equation presented earlier also predicts that the influence of cellular amino acids on membrane potential should be a function of the cellular Na^+ concentration. This prediction is supported by the report that glycine efflux increases with increasing cellular Na^+ [14]. If the increased amino acid efflux is accompanied by an increase in Na^+ efflux via co-transport when the cells are introduced into amino acid-free media, then the influence of cellular amino acid on the membrane potential should be greatest with high cell Na^+ and much less at low cell Na^+. To test for this influence cells were first equilibrated in the presence and absence of 3 mM 2-aminoisobutyrate at a 1 : 320 dilution at 37°C for 30—45 min. The samples were then divided into various aliquots, centrifuged, and resuspended at 1 : 60 dilution in (1) K^+-Ringer (2) K^+-free Na^+-Ringer or (3) mixtures of (Na^+ + K^+)-Ringer all containing 10 mM glucose and incu-

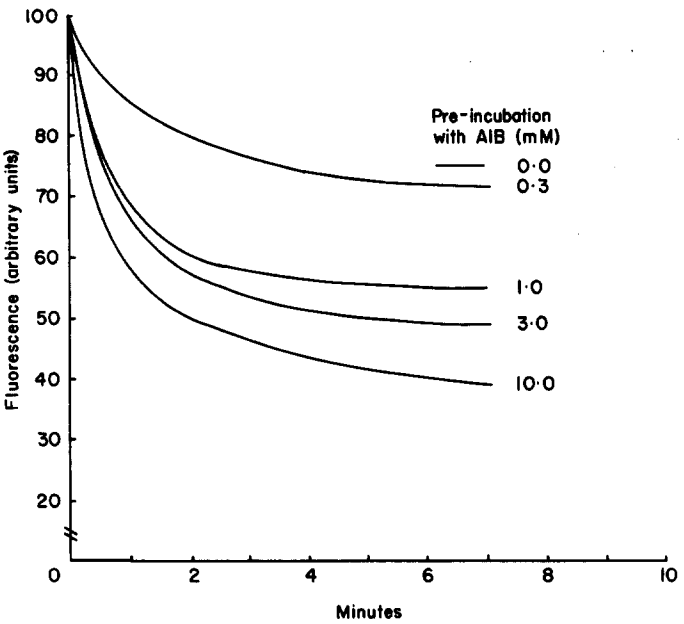


Fig. 5. Cells were diluted to 1 : 320 in Na^+-Ringer containing either 0, 0.3, 1.0, 3.0, or 10.0 mM 2-aminoisobutyric acid and incubated at 37°C. After 30 min, the suspensions were centrifuged and resuspended in Na^+-Ringer in the absence of 2-aminoisobutyric acid. Dye was then added to aliquots and the fluorescent intensity recorded.

bated 1.5 h at 6°C to alter the ($Na^+ + K^+$) levels in the cells. After 1 or 2 h of cold storage the concentration of 2-aminoisobutyrate in the cells stored in K^+-Ringer was somewhat lower than the concentration in the cells stored in K^+-free Na^+-Ringer. This difference is due to the greater swelling of the cells in K^+ medium, for the amount of 2-aminoisobutyrate per mg protein was approximately equal. After cold storage the cells were pelleted and all were resuspended at a dilution of 1 : 320 in an amino acid-free medium of the following composition: 120 mM NaCl, 30 mM KCl, 1.5 mM $MgSO_4$, 10 mM sodium phosphate buffer, pH 7.4, 10 mM glucose and 1 mM ouabain. The steady levels of fluorescence attained in the initial stage were then measured at 33—34°C. The data presented in Table II indicate that (1) cells cold-stored in Na^+ ($[Na]_i > 50$ mM) with 2-aminoisobutyrate are always hyperpolarized compared to cells cold-stored without 2-aminoisobutyrate. (2) In cells stored in K^+ medium and possessing low cellular Na^+ (<20 mM $[Na]_i$) cellular 2-aminoisobutyrate content does not influence the potential difference. (3) In cells which have been depleted of amino acids, alteration of cellular Na^+ and K^+ composition does not influence the membrane potential upon dilution of the cells under the conditions employed in these experiments. These experiments show that the hyperpolarizing effect of cellular amino acids is a function of cell Na^+ and conversely that the hyperpolarizing phase of dilution in cells with high Na^+ is associated with cellular amino acids.

Na^+ efflux from cells with high 2-aminoisobutyrate content was compared with that from cells with low amino acid content. Cells for this experiment

TABLE II

FLUORESCENT INTENSITY AS A FUNCTION OF CELLULAR 2-AMINOISOBUTYRATE, Na^+ AND K^+ CONCENTRATION

Cells (1 : 320 dilution) were pre-equilibrated for 30 min in Na^+-Ringer + 3 mM 2-aminoisobutyrate (AIB) at 37°C. Aliquots of the cells were resuspended (1 : 60 dilution) in either cold (6°C) K^+-free Na^+-Ringer, K^+-Ringer (series 1) or mixtures of these two solutions (series 2) for 1.5 h. Cells were then suspended in 120 mM NaCl, 30 mM KCl, 1.5 mM $MgSO_4$, 10 mM sodium phosphate buffer at pH 7.4, 10 mM glucose and 1 mM ouabain at 33—34°C. Dye (final concentration equals $3.0 \cdot 10^{-6}$ M) was added and the steady level of fluorescent intensity recorded ($n = 3 \pm$ S.E).

Cellular concentrations (mmol/l cell H_2O)			Fluorescent intensity (arbitrary units)
K^+	Na^+	AIB	$n = 3 \pm$ S.E.
Series 1			
69	96	0	23.5 ± 0.2
79	64	33	15.1 ± 0.5 *
175	17	0	25.3 ± 0.2
143	15	27	23.4 ± 0.7
Series 2			
30	95	0	23.4 ± 0.3
68	64	0	22.3 ± 0.6
89	40	0	23.1 ± 0.6
119	16	0	23.4 ± 0.4

* Significantly different at the 1% level.

were first equilibrated for 30 min at 37°C in the presence and absence of 3 mM 2-aminoisobutyrate at a 1 : 320 dilution. The cells were then centrifuged and brought to a 1 : 20 dilution with cold Na^+-Ringer containing $^{22}Na^+$ as a tracer. After cold storage for 1 h, the cells were washed and placed in a Na^+-free choline/Tris/MOPS medium containing ouabain. In this medium, Na^+-Na^+ exchange and Na^+ extrusion via the $Na^+ : K^+$ pump were eliminated. The data shown in Fig. 6 demonstrate that the rate constant for $^{22}Na^+$ efflux from cells containing 2-aminoisobutyrate is 40% higher than that of cells depleted of amino acids.

$Na^+ : K^+$ pump activity

While the transient hyperpolarization followed by depolarization seen during dilution and washing of the cells appears to be associated with changes in cellular amino acid level, conditions leading to changes in cellular cation composition and/or ATP also appear to alter the membrane potential by changes in Na^+ pump activity. Hyperpolarization (decrease in fluorescent intensity) was observed previously [4] when cells depleted of ATP by incubation with the respiratory inhibitor, rotenone, were treated with glucose provided that the cellular Na^+ levels had been raised during the incubation period with rotenone. Ouabain inhibited this hyperpolarization in cells given glucose but had no effect in the absence of glucose. Since cellular Na^+ can also be raised by storing cells in the cold [15], a series of experiments was performed to see if cells with elevated Na^+ levels would hyperpolarize when returned to a warmer (20—37°C) temperature. Ouabain-sensitive hyperpolarization would be consistent with an electrogenic Na^+ pump and such changes have been reported by other

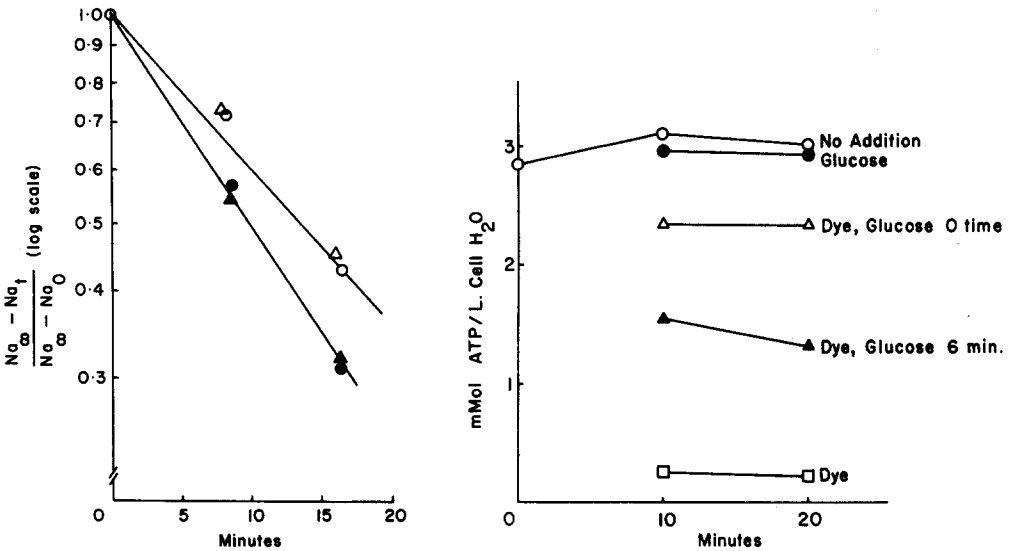


Fig. 6. Cells were diluted to 1 : 320 in Na^+-Ringer in the presence and absence of 3 mM 2-aminoisobutyric acid and incubated at 37°C. After 30 min, the suspensions were centrifuged and the cells were resuspended in cold Na^+-Ringer containing 10 mM D-glucose in the presence and absence of 3 mM 2-aminoisobutyric acid (same as initial) plus $^{22}Na^+$ as a tracer at a 1 : 20 dilution and stored at 4°C for 1 h. Cells were then analyzed for $^{22}Na^+$ efflux (see Methods) into choline/Tris/MOPS/Ringer containing 10 mM glucose plus 1 mM ouabain. Two different-experiments (circles and triangles) are shown (representative of 10 experiments). Each point is the average of four determinations. With closed symbols, cells contain 2-aminoisobutyrate; with open symbols, they do not.

Fig. 7. Cells were diluted to 1 : 20 in Na^+-Ringer and stored at 4°C for 2 h. They were then diluted to 1 : 320 in Na^+-Ringer and incubated at 20°C. Conditions: ○, no further additions; ●, D-glucose (10 mM) added at zero time; △, dye ($3 \cdot 10^{-6}$ M) plus D-glucose (to 10 mM) added at zero time; ▲, dye added at zero time and D-glucose added at 6 min; and □, dye ($3 \cdot 10^{-6}$ M) alone at zero time. Aliquots were taken at intervals and analyzed for cellular ATP.

investigators [16,17]. In the early experiments cold-stored cells did not appear to become hyperpolarized on warming and ouabain had no effect on the membrane potential even though the cellular Na^+ was raised during cold storage. Upon addition of glucose after the cold storage, however, hyperpolarization was seen and this hyperpolarization was blocked or reversed by the addition of ouabain. Since measurements showed that the cells contain 2—3 mM ATP during the incubation period following cold storage, the question arose as to why the addition of glucose was required to observe electrogenic activity of the pump. The answer to this question is seen in Fig. 7. Particularly after cold storage, the addition of dye to estimate potentials causes cellular ATP levels to fall rapidly; the addition of glucose decreased the drop in the ATP level. In the absence of dye, cells retain nearly normal ATP levels. Lactic acid production upon the addition of glucose is increased in the presence of dye (data not shown) much as it is when the cells have been pretreated with rotenone. The level of ATP found in cells with dye plus glucose varies according to the time when the glucose was added with respect to the time of addition of dye. The highest levels of cellular ATP in the presence of dye were observed when glucose was added before the dye.

Cells stored in the cold in the presence of 10 mM glucose showed marked hyperpolarization when they were warmed provided that cellular Na^+ was substantially raised (Fig. 8). The addition of 1 mM ouabain then depolarized the cells. With time at 37°C, cellular Na^+ levels were reduced and the cells again became depolarized returning to a potential difference that approached the level recorded prior to cold storage (Fig. 9). In contrast, cells maintained in the cold without glucose showed variable hyperpolarization when they were rewarmed and glucose was added. The variability was probably due to the variable level of ATP and hence, variable pump activity, in cells treated with dye after cooling and rewarming in the absence of glucose.

If the hyperpolarization inhibited by ouabain is dependent on $(Na^+ + K^+)$-ATPase activity, then it should also require the presence of K^+ in the medium in addition to glucose. To test for this requirement cells preincubated to deplete them of amino acids were suspended in cold K^+-free Na^+-Ringer plus glucose and stored at 4°C for 1 or 2 h. Subsequently, after warming them to 37°C dye was added and the fluorescent intensity was measured before and after the addition of K^+. The addition of increasing K^+ concentrations resulted in a decrease in fluorescent intensity (Fig. 10) indicating a hyperpolarization.

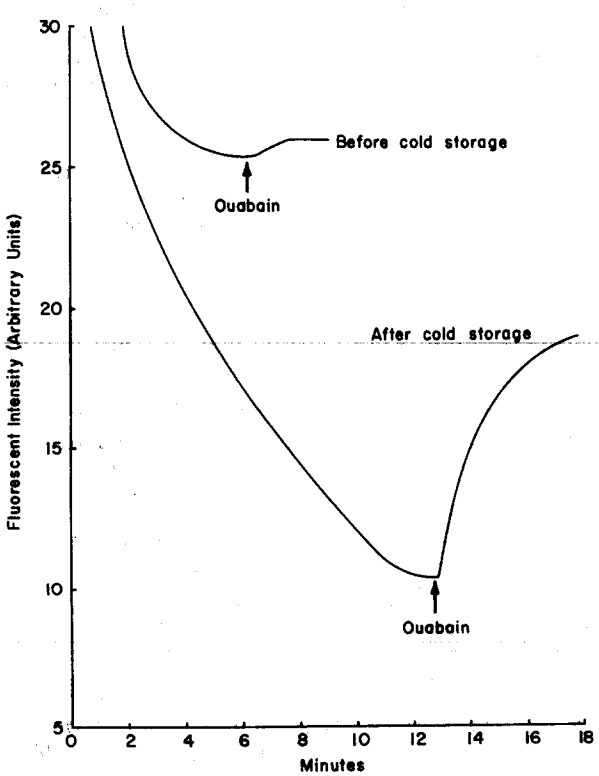


Fig. 8. Cells were diluted 1 : 320 in Na^+-Ringer and incubated at 37°C for 30 min. After 30 min, glucose (10 mM) was added and the sample was divided. Dye was then added to aliquots of one portion and the fluorescent intensity recorded (curve marked Before cold storage) at 37°C. The other portion was stored at 4°C for 2 h. Aliquots of this portion were then warmed to 37°C; dye was added and fluorescent intensity recorded (curve marked After cold storage). Ouabain (1 mM) was added where indicated.

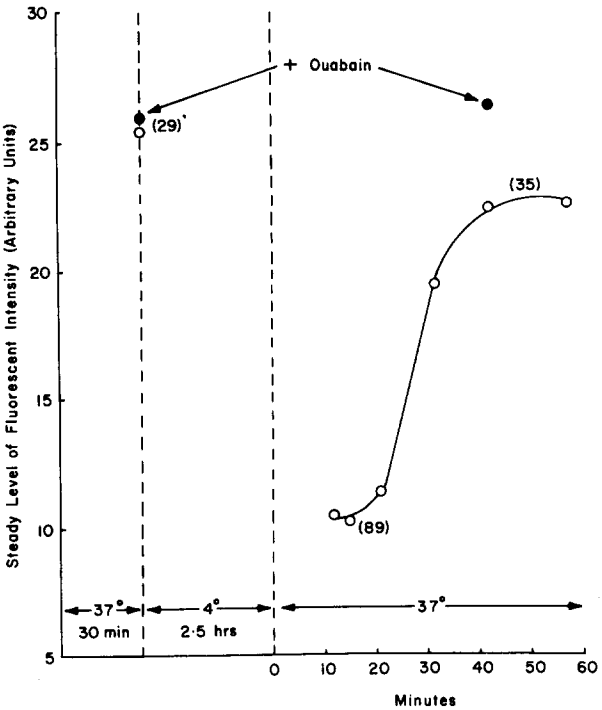


Fig. 9. Cells were diluted 1 : 320 in Na^+-Ringer and incubated at 37°C for 30 min. After 30 min glucose (10 mM) was added. Dye was then added to an aliquot and the steady level of fluorescent intensity recorded in the presence and absence of 1 mM ouabain. The remainder of the cell suspension was stored at 4°C for 2.5 h after which the suspension was re-incubated at 37°C. At intervals dye was then added to aliquots and the steady level of fluorescent intensity recorded in the presence (●) or absence (○) of 1 mM ouabain. Numbers in parentheses indicate the cellular Na^+ levels at the time recorded.

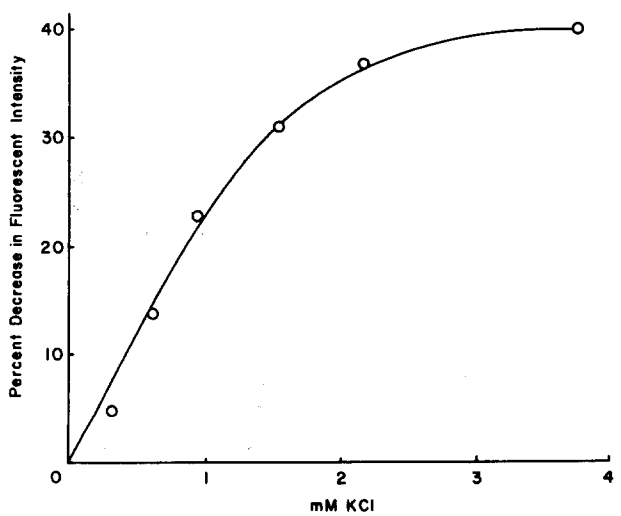
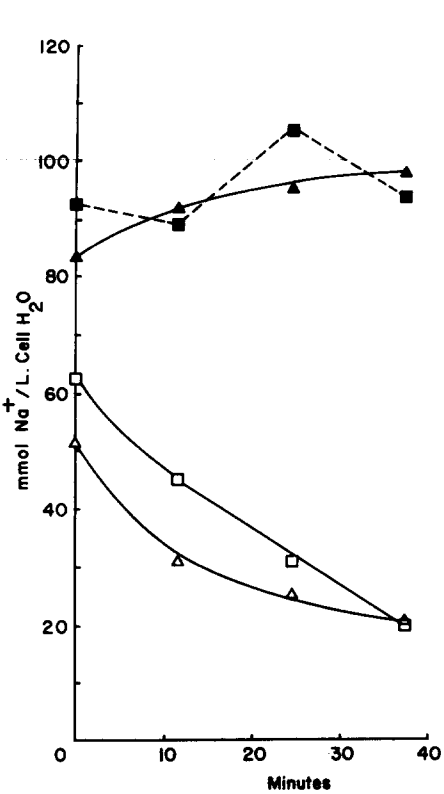
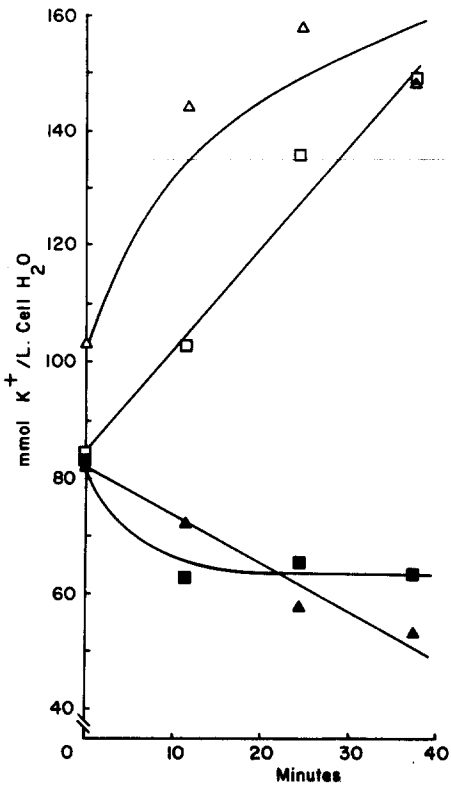


Fig. 10. Cells were preincubated at 1 : 320 for 30 min at 37°C and then transferred to K^+-free Na^+-Ringer containing 10 mM glucose and incubated for a further 1—2 h at 4°C. After warming them to 37°C, dye was added and fluorescence measured. Additions of KCl were made and the percentage decrease in fluorescent intensity was measured.





Figs. 11 and 12. Cells were diluted to 1 : 20 with K^+-free Na^+-Ringer stored at 4°C for 1.5 h. The cells were then diluted to 1 : 320 with Na^+-Ringer and incubated in the presence and absence of 1 mM ouabain, dye and glucose. △, control cells, no further additions; ▲, +1 mM ouabain; □, dye ($3 \cdot 10^{-6}$ M) and glucose to 10 mM; ■, dye + glucose + ouabain. Cells were analyzed at intervals for Na^+, K^+ and water content.

Ouabain (1 mM) reversed or prevented the hyperpolarization if added prior to K^+. In the absence of K^+ there was no change in fluorescence with ouabain. Similar results were obtained with cells preincubated in K^+-free Ringer at 37°C.

While the measurements of fluorescence implied that the $Na^+ : K^+$ pump was functional in the presence of dye, several experiments were performed to demonstrate cation movements associated with pump activity under conditions comparable to those used for the fluorescence studies particularly with cold-stored cells. Net loss of Na^+ and gain of K^+ were recorded when cold-stored cells were incubated in dye plus glycose (Figs. 11 and 12). It may be seen that in the presence of dye plus glucose Na^+ and K^+ are restored to normal levels, albeit at a slower rate. Ouabain blocked the extrusion of Na^+ and uptake of K^+ in both control cells and cells with dye plus glucose. When dye was added without glucose, the extrusion of Na^+ and uptake of K^+ was not seen or was very small (data not shown).

An ouabain-sensitive $^{22}Na^+$ efflux was also seen in the presence of dye plus glucose (Fig. 13) but not with dye in the absence of glucose (data not shown) in cold-stored cells. In accord with the observations on changes in cation com-

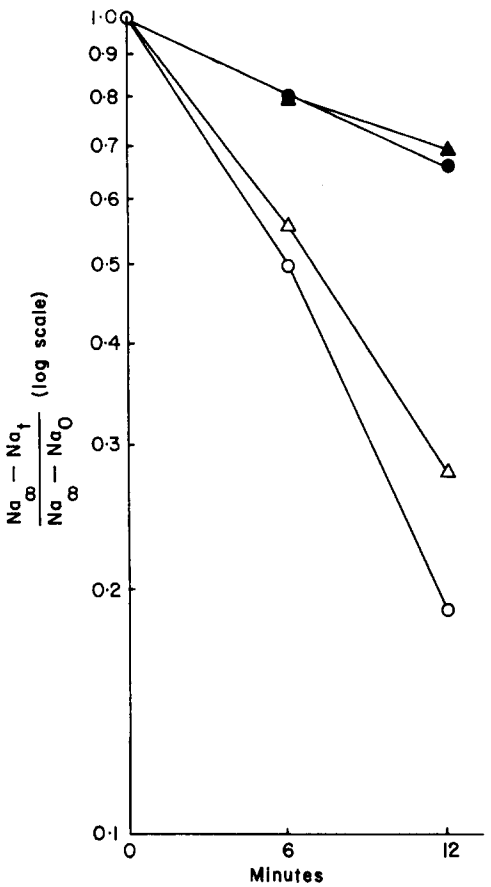


Fig. 13. Cells were preincubated in Na^+-Ringer at 1 : 320 for 30 min at 37°C. They were then centrifuged and resuspended at 1 : 20 cold K^+-free Na^+-Ringer containing 10 mM glucose and $^{22}Na^+$ as a tracer. After storage at 6°C for 2 h, the cells were washed and resuspended at 1 : 320 in Na^+-Ringer containing 10 mM glucose at 37°C with and without 1 mM ouabain. Dye ($3 \cdot 10^{-6}$ M) was added immediately to two aliquots (and an equivalent amount of ethanol was added to the other two). Sampling was initiated after 8 min incubation. ○, ethanol alone; ●, ethanol plus ouabain; △, dye alone; ▲, dye plus ouabain.

position, ouabain-sensitive $^{22}Na^+$ loss was somewhat slower in the presence of dye (plus glucose). The ouabain-insensitive $^{22}Na^+$ efflux, however, was not influenced by the dye.

Discussion

The evidence presented above indicates that the membrane potential of the Ehrlich ascites tumor cell varies with cellular Na^+, the amino acid composition and with the activity of the $Na^+ : K^+$ pump, in accordance with Eqn. 1 as presented by Geck et al. [1].

We have previously reported [4] that the membrane potential estimated by the null point method after pre-equilibration, i.e. after incubation of the cells at a 1 : 320 dilution at 37°C for 30 min (or at 20°C for 1 h) was in reasonable agreement with the potentials measured by Lassen et al. [18] with electro-

physiological techniques and those calculated from the chloride ratio assuming that Cl^- is distributed equally throughout the cell water [4,18,19]. Without this pre-equilibration, however, the cells were temporarily hyperpolarized when diluted. Two possible causes of this initial hyperpolarization upon dilution were considered. First, there was the possibility that an electrogenic $Na^+ : K^+$ pump was activated by a gain in cellular Na^+ during the washing procedure. With time as cellular Na^+ levels were returned to normal, the activity of the pump would be expected to decline and a depolarization observed. Second, the dilution required for the fluorescence studies could result in a hyperpolarization produced by the enhanced efflux of Na^+ co-transported with endogenous amino acids. As the endogenous pool of amino acids was depleted, Na^+ efflux would diminish and the cells would depolarize. Although in either case the hyperpolarization would depend upon cellular Na^+, the two possibilities could be distinguished on the basis of their dependence on ATP and their inhibition by ouabain. The electrogenic activity of the $Na^+ : K^+$ pump would require ATP and should be inhibited by ouabain while the influence of the co-efflux of Na^+ and amino acids on the membrane potential should be independent of cellular ATP and insensitive to ouabain. Since ATP levels fell sharply in the presence of the dye, the requirement of ATP for the development of the potential could be discerned as a requirement for glucose *. Since the transient initial hyperpolarization upon dilution was seen both in the absence of glucose, and in the presence of ouabain, the electrogenic activity of the $Na^+ : K^+$ pump was eliminated as a factor here. On the other hand the hypothesis that Na^+-coupled efflux of endogenous amino acids could produce the initial hyperpolarization was supported by several lines of evidence. (1) The Ehrlich cells contain higher concentrations of amino acids than does the fluid in which they are suspended in the mouse. When this fluid is replaced by an amino acid-free medium, a large loss of these amino acids was measured especially upon dilution of the cells to 1 : 320. This amino acid loss coupled to Na^+ could account for the initial hyperpolarization and subsequent depolarization observed with the cells upon dilution. It has been shown [14] that the efflux of amino acids is increased by cellular Na^+ suggesting that the efflux of amino acids is coupled to Na^+. (2) It was also noted (data not shown) that if the cells were suspended in Na^+-Ringer containing the mixture of amino acids found in ascites fluid, the cells were not hyperpolarized and underwent no depolarization on dilution. (3) The magnitude of the membrane potential was shown to vary with the size of the amino acid pool (in these experiments largely 2-aminoisobutyrate). While the influence of the amino acid pool was dependent on cellular Na^+, ouabain did not alter this influence, and the effects of cellular amino acids were seen in the absence of glucose, i.e. under conditions where ATP levels were low. (4) Na^+ efflux was enhanced by cellular amino acids in the presence of ouabain. This Na^+ movement could be responsible for the hyperpolarization observed. (5) Finally, cells incubated at a dilution of 1 : 320 or 1 : 20, have identical Na^+ and K^+ levels (not shown). Hence, the differences in degree of polariza-

* According to Waggoner [20], the dye inhibits activity at site 1 in the mitochondrion. The data presented above, however, indicate that cellular ATP can be maintained at nearly normal levels in the presence of the dye provided that glucose is available.

tion upon dilution cannot be due to differential activation of the $(Na^+ + K^+)$-ATPase because of differences in cellular cations.

Although eliminated from consideration as a cause of the initial hyperpolarization upon dilution of cells, the activity of the $Na^+ : K^+$ pump was shown to have an important influence on membrane potential under different experimental conditions. The evidence for electrogenic activity of the pump is based on a similarly of the requirements for hyperpolarization seen in these experiments and the known activity of the $(Na^+ + K^+)$-ATPase (pump), i.e. the simultaneous presence of cellular Na^+ and ATP and extracellular K^+. Ouabain, a specific inhibitor of the $Na^+ : K^+$ pump, inhibited or reversed the hyperpolarization. Attempts to correlate the magnitude of the membrane potential and the cellular Na^+ content were hampered by the fact that cellular Na^+ changes rapidly when pumping is restored after cold storage and both the fluorescent studies and preparation of the cells for cation analyses required several minutes. It was observed, however, that hyperpolarization resulting from pump activity was small when cellular Na^+ was in the range 25—30 mM and large when cellular Na^+ was in the range 60—70 mM (in some cases hyperpolarization appeared to occur in the range of 40—50 mM Na^+). The magnitude of the potential difference at 37°C in cells with elevated cellular Na^+ (after cold storage) was estimated on several occasions in the presence of glucose using the null point method [4,21]. These potentials were in the range —44 to —63 mV. While it is possible that the dye causes some inhibition of the $Na^+ : K^+$ pump directly, cells were able to show net transport of Na^+ and K^+ in the presence of dye plus glucose.

In summary, the membrane potential of Ehrlich ascites tumor cells can be influenced by amino acid (Na^+ co-transport) efflux and by the activity of the $Na^+ : K^+$ pump. These factors need to be taken into consideration when the energetics and kinetics of ion and amino acid transport are discussed.

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