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Evidence for Activation of an Active Electrogenic Proton Pump in Ehrlich Ascites Tumor Cells during Glycolysis

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Summary. The addition of glucose to a suspension of Ehrlich ascites tumor cells results in rapid acidification of the extracellular medium due to lactic acid production. The nature of the H^+ efflux mechanism has been studied by measuring the time course of the acidification, the rate of proton efflux, the direction and relative magnitude of the H^+ concentration gradient, and the voltage across the membrane. Using the pH-sensitive dye acridine orange, we have established that after addition of 10 mM glucose an outward-directed H^+ concentration gradient develops. As the rate of glycolysis slows, the continued extrusion of H^+ reverses the direction of the H^+ concentration gradient. Changes in absorbance of the voltage-sensitive dye diethyloxadicarbocyanine iodide (DOCC), and changes in the distribution of the lipid permeant cation tetraphenyl phosphonium, showed a dramatic and persistent hyperpolarization of the membrane voltage after glucose addition. The hyperpolarization was prevented by the protonophore tetrachlorosalicylanalide (TCS) and by valinomycin, but not by the neutral-exchange ionophore nigericin. Inhibitors of lactate efflux were found to reduce the rate of acidification after glucose addition but they had no effect on the magnitude of the resulting hyperpolarization. On the basis of these and other data we suggest that an active electrogenic pump mechanism for H^+ efflux may be activated by glucose and that this mechanism operates independently of the lactate carrier system.

Key words: H^+ secretion, acidification, glycolysis, Ehrlich ascites tumor cell, ion transport, membrane voltage lactate transport

It has been recognized for many years that tumor cells exhibit rapid aerobic glycolysis in the presence of a sufficient source of glucose (Warburg, 1956). This characteristic is shared by Ehrlich mouse ascites tumor cells, which in the presence of glucose, exhibit rapid formation of lactic acid and a resulting acidification of the cytoplasm and extracellular medium (Chance & Hess, 1956; Racker, 1956; Warburg, 1956; Poole, 1967; Thomas, Buchsbaum, Zimniak & Racker, 1979). For this reason most transport studies with Ehrlich ascites cells have employed glucose-free media. Although glucose transport and metabolism have been the subjects of numerous reports, the mechanisms by which lactate and protons produced by glycolysis are transported out of the cell have received less attention. In the present studies we have examined, in particular, proton efflux from the cells; but before introducing our approach it is necessary to consider some characteristics of glucose uptake and metabolism in the Ehrlich cell.

As shown by Warburg (1956), in the presence of sufficient glucose, respiration is inhibited and glycolysis results in rates of energy production approximately equivalent to those during purely respiratory metabolism. The glycolytic capacity of the tumor cells may be responsible for the low glucose concentration (<0.2 mM) found in the ascitic fluid *in vitro* (Fishman & Bailey, 1974), although it may also be attributable in part to a compromised rate of glucose delivery by the systemic circulation relative to the dense cell suspension in the peritoneal cavity (Warburg, 1956).

The effect of glucose on glycolysis is bisphasic. Within the first minute after glucose addition respiration is actually stimulated together with glycolysis. After 1 min both respiration and glycolysis are markedly reduced (Chance & Hess, 1956). Part of this inhibition may be the consequence of acidification of the cytoplasm and extracellular medium. Racker (1956) showed that at high glucose concentrations, the medium pH could be reduced to as low as 5.0. Acidification of the medium occurred in parallel with lactate efflux and a reduced cytoplasmic pH (Poole,

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1967; Thomas et al., 1979). It has been proposed that lactic acid leaves the cell down its concentration gradient in the undissociated form (Poole, 1967; Thomas et al., 1979), and Thomas et al. (1979) have shown that, at least initially, the intracellular pH is less than that of the medium which provides the necessary gradient for efflux. Spencer and Lehninger (1976) demonstrated that lactate transport in the Ehrlich cell occurs via a saturable mechanism, which is shared by several monocarboxylic acids and which can be inhibited by mersalyl. Furthermore, they proposed that lactate transport is electroneutral and involves either symport together with H^+ or antiport in exchange for OH^- or bicarbonate.

Even in the absence of glucose Heinz, Geck and Pfeiffer (1977) have shown that Ehrlich cells acidify the medium. Furthermore, in spite of the inside-negative transmembrane voltage, the intracellular pH appears to be higher than the extracellular, and Geck, Pietrzyk, Heinz and Pfeiffer (1978) have suggested the presence of an active proton pump which produces net proton efflux against a small concentration difference and an electrical potential difference. In spite of relatively low pump rates, Geck et al. (1978) proposed that the proton electrochemical potential could be maintained due to the low permeability of the membrane to protons. The pump itself could involve either an electrogenic H^+ transport, or electroneutral H^+-K^+ antiport (Heinz et al., 1977).

In the present studies, we have examined the time course, direction and magnitude of the transmembrane pH difference and the rate of net H^+ efflux during glycolysis using direct recording of extracellular pH, voltage- and pH-sensitive dyes, and measurement of the rate of base addition required to maintain a constant medium pH (pH-stat method). Using the pH-sensitive dye acridine orange, we have established that immediately after glucose addition an outward-directed H^+ concentration gradient was produced as an apparent consequence of a glycolytic rate in excess of lactate and H^+ exit. However, as the rate of glycolysis slowed an inward-directed H^+ concentration difference developed with continued net H^+ efflux. Using a voltage-sensitive dye, we established further that glucose addition results in a dramatic and persistent hyperpolarization of the transmembrane voltage which was abolished by the protonophore tetrachlorosalicylanalide (TCS) and by valinomycin but not by the neutral exchange-ionophore nigericin. On the basis of these and other data we suggest that an active electrogenic H^+ pump mechanism may be activated during glycolysis.

Materials and Methods

The general methodology used by this laboratory for transport studies in Ehrlich mouse ascites tumor cells has been described previously (Schafer & Heinz, 1971; Schafer, 1977). The cells used in these studies were derived from the hyperdiploid Ehrlich-Lettré line and were maintained by weekly intraperitoneal injection of ascites from previously injected animals. Cells were harvested for transplantation or experimentation 6–9 days after injection. For experiments, they were aspirated into a syringe and transferred to a buffered solution (referred to as Na-KRP) containing (in mM): 128 NaCl, 10 Na_2HPO_4/NaH_2PO_4 (pH 7.4), 20 KCl and 1.5 $MgSO_4$. For the initial collection and dilution of ascitic fluid, 100 units of sodium heparin were added to each 30 ml of buffer.

The cells were centifuged (at ca. $50 \times g$) and resuspended two times in Na-KRP. They were then suspended in the same medium to a cytocrit of 5% and kept at room temperature for no more than 1 hr before use. Prior to each experimental incubation, an aliquot of suspension was centrifuged and the cells were resuspended in a buffer solution (referred to as low-Na^+) consisting of (mM): 102 choline chloride, 23 NaCl, 10 Na_2HPO_4/NaH_2PO_4 (pH 7.4), 20 KCl and 1.5 $MgSO_4$. In those experiments in which the time course of medium pH was followed, only 1.5 mM phosphate was used and the remainder was replaced isosmotically by NaCl. In all experiments the final cytocrit was 5%, and the cells were incubated for 10 min at 37° C before measurements were made. Additions of all substates and other biochemicals to the cell suspension were made using concentrated stock solutions in the same buffer solution so that the final suspension volume varied by less than 1% during the course of an experiment.

Time Course of Extracellular pH

Extracellular pH was measured by immersing a small combination pH electrode (Orion Research, Cambridge, Mass., Model 91-03) in 5 ml of Ehrlich ascites cell suspension in the 1.5 mM phosphate buffer solution (*see above*). The pH was followed using an Orion Research Model 701A pH meter connected to a strip-chart recorder.

Rate of Acidification at Constant pH

We measured the rate of proton efflux into the extracellular medium at a constant pH of 7.4 by means of a Radiometer-Copenhagen (London Co., Cleveland, Ohio) pH-stat system. The acidification rate was quantified from the rate of addition of low-Na-KRP buffer to which 0.02 N NaOH had been added. Addition of the basic solution from a model ABU12 Autoburette into the stirred, temperature-regulated (37° C) chamber was controlled by a TTT60 titrator and recorded as a function of time on an REC61/REA110 Servograph connected to a PHM64 pH meter. After each experiment the entire cell suspension was collected and the cells were centrifuged, frozen and lyophilized. The rate of H^+ efflux was then normalized per measured g dry weight of cells in the suspension.

Dye Distributions

Measurements of acridine orange and diethyloxadicarbocyanine (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. Acridine orange absorbance was measured at 490 nm as a differential absorbance relative to a reference wavelength of 530 nm. DOCC absorbance was measured at 580 nm relative to 630 nm. Final dye concentrations in the suspensions were 15 μM acridine orange or 10 μM DOCC. In both cases the cytocrit was carefully maintained at 5%.

After addition both dyes showed rapidly decreasing absorbance due to entry into the cells with resulting quenching (Waggoner, Wang & Tolles, 1977; Dell'Antone, 1979). The absorbance of both

dyes reached a stable minimum within 5 min at which point glucose or other substrates were added.

Distribution of Tetraphenyl Phosphonium (TPP^+)

As a lipid-permeable cation, the steady-state distribution of TPP^+ may be used as an index of the membrane voltage (Heinz, Geck & Pietrzyk, 1975). The uptake of TPP^+ was measured under varying conditions by adding 5–10 μM TPP^+ with ^3H-TPP^+ label to the normal Na-KRP medium. After incubation for varying times at 37° C, cells were separated by centrifugation, and the pellets were frozen, lyophilized and extracted as described previously (Schafer and Heinz, 1971; Schafer, 1977). The activity of ^3H in the extract was corrected for extracellular ^3H-TPP^+ by using ^{14}C-sorbitol as an extracellular volume marker (Schafer, 1977).

Sources of Biochemicals

Nigericin sodium was kindly provided by Dr. R.J. Hosley from Lilly Research Laboratories (Indianapolis, Ind). Tetrachlorosalicylanalide (TCS) was a gift of Dr. W.H. Hamilton (University of Aberdeen, Aberdeen, U.K.), and ^3H-tetraphenylphosphonium bromide was a gift of Dr. R. Kaback (Roche Institute of Molecular Biology, Nutley, N.J.). Other biochemicals were obtained in purest available grade as follows: D- and L-glucose, 2-deoxy-D-glucose, L-lactate, L-alanine and valinomycin from Sigma Chemical Co. (St. Louis, Mo.); DOCC (Custom Synthesis, lot no. C3D) and acridine orange (lot. no. B6B) from Eastman Kodak Co. (Rochester, N.Y.); L-pyruvate from Mallinkrodt Inc., (St. Louis, Mo.).

Statistics

Single experiments presented as figures were repeated at least 3 times with identical results. Standard deviations are indicated for results presented as mean values.

Results

The series of experiments to be described in this section was designed to examine the process of extracellular fluid acidification observed when Ehrlich ascites tumor cells are incubated in the presence of glucose (Racker, 1956; Warburg, 1956). Five basic types of measurements were made: (1) the rate of medium acidification; (2) proton efflux from the cells at constant medium pH (pH-stat method); (3) the distribution of the pH-sensitive dye acridine orange; (4) the distribution of the voltage-sensitive dye diethyloxadicarbocyanine iodide (DOCC); and (5) the distribution of the lipid-permeant cation tetraphenylphosphonium (TPP^+).

These measurements were made in the presence and absence of glucose, and in conjunction with agents known to modify the ionic conductivity and/or permeability properties of the cell membrane. Tetrachlorosalicylanilide (TCS) was used to increase specifically the membrane conductance to protons (Zimmer, 1977). Valinomycin was used as a K^+-specific ionophore in order to convert the transmembrane voltage to the potassium equilibrium voltage by increasing the membrane K^+ conductance (Haydon & Hladky, 1972). Nigericin was used as a neutral exchange ionophore which allows electroneutral exchange among Na^+, K^+ and H^+ (Harris & Pressman, 1967; Haydon & Hladky, 1972). With these methods and membrane-active agents, we have established the rate of medium acidification, the direction of transmembrane proton concentration gradients and changes in transmembrane voltage which occur after the addition of glucose.

DOCC has been used previously as an indicator of transmembrane voltage (Rabon, Chang & Sachs, 1978). As a positively charged carbocyanine compound it tends to accumulate rapidly in the cell interior or in organelles where aggregation reduces its absorbance (Waggoner et al., 1977; Rabon et al., 1978). Thus hyperpolarization of the inside-negative membrane voltage reduces the DOCC absorbance in a cell suspension.

Acridine orange is a weak base that distributes in response to a transmembrane pH gradient accumulating on the low pH side with quenching of its absorbance at 490 nm; however, the absorbance may also be decreased by changes in binding to anionic polymers (Dell'Antone, Colonna & Azzone, 1972). Nevertheless, acridine orange has been shown to be an excellent and rapidly responding indicator of changes in transmembrane pH gradients in a variety of systems (Schuldiner, Rottenberg & Avron, 1972; Rabon et al., 1978; Dell'Antone, 1979; DiBona, Ito, Berglindh & Sachs, 1979).

Medium Acidification

In the first series of experiments we continuously recorded the pH of a weakly buffered (1.5 mM Na_2HPO_4/NaH_2PO_4, initial pH 7.4) low-Na^+ solution. Cells were suspended in the medium to a cytocrit of approximately 5%. Fig. 1 shows the results of three separate experiments. In each experiment, the cells were initially placed in identical glucose-free buffer solutions. Under these conditions there was a slow decrease in pH with time which ranged from 0.005 to 0.017 pH units/min. In the experiment shown by the solid line, when glucose was added at time zero there was an immediate increase in the rate of medium acidification to an initial rate ranging from 0.20 to 0.40 pH units/min which began to slow after 30–60 sec to a steady rate of 0.04 to 0.10 pH units/min. Acidification of the bathing solution continued over a 40-min period and reached a limiting external pH ranging from 5.3 to 5.7 at which acidification ceased. The cessation of acidification at ~pH 5.5 did not appear to result from gross damage to the cells because samples examined at this time point showed that less than 5% of the cells were stained with trypan blue.

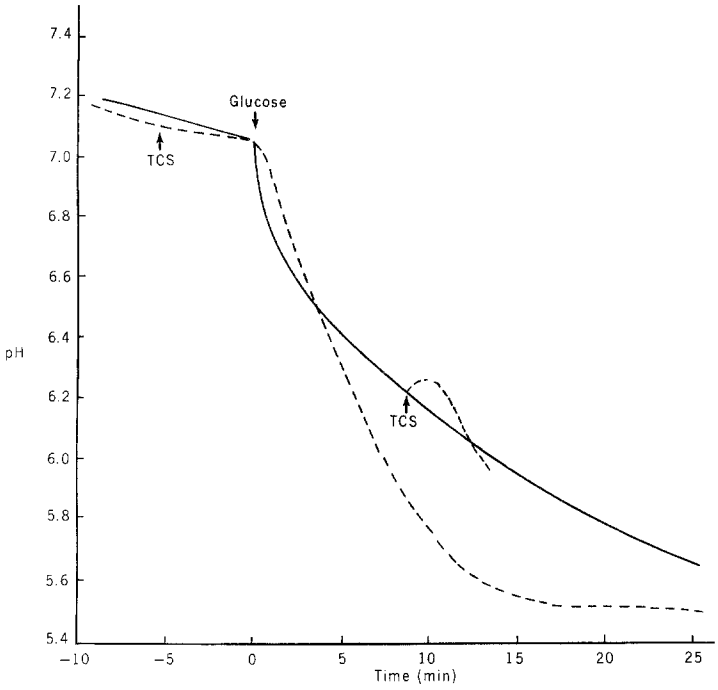

Fig. 1. Acidification of the external medium after glucose addition. The medium pH is plotted as a function of time, with the point of glucose addition taken at zero time. Three experiments are shown. In one experiment (solid line) glucose was added and the pH recorded over a 25-min period. In the second experiment 12 μM TCS was added 10 min after glucose addition (upper broken curve). In the third experiment (lower broken curve), 12 μM TCS was added 5 min before glucose

In several experiments 5 to 12 μM TCS was added after glucose addition had reduced the medium pH to values in the range of 6.0 to 6.8. As shown by the dashed curve in Fig. 1, at approximately 10 min, TCS resulted in a transient alkalinization of the medium. We interpret this observation to indicate that at the point of TCS addition the cell had a lower proton concentration than the medium, so that the increased H^+ conductance produced by TCS resulted in H^+ backflux into the cells, shown by a transient alkalinization ranging from 0.05 to 0.10 pH units.

Addition of TCS to the medium 5 min prior to glucose addition resulted in about the same initial rapid rate of acidification, 0.2–0.3 pH units/min, as shown by the dashed curve in Fig 1. However, in this case the rate did not tend to slow markedly with time and the same limiting pH of 5.5 was reached within only 10–15 min. When the same experiments shown in Fig. 1 were carried out at room temperature, or in Na-KRP rather than low Na-KRP, the results were virtually identical both temporally and in the rate and extent of pH change. In experiments which are not shown, addition of other metabolic substrates including 10 mM acetate, alanine, and lactate did not change the basal rate of acidification, but addition of 10 mM glucose did produce acidification as in Fig. 1 even after addition of these other substrates. Furthermore, neither the basal rate of acidification nor the glucose-stimulated rate appeared to be dependent on respiration since prior addition of 20 μM cyanide or 0.5 mM amytal had no effect on either.

Table 1. Rate of H^+ efflux before and after addition of 10 mM glucose to the medium

Time relative to glucose addition (min)	H^+ efflux ($\mu mol\ min^{-1}\ g^{-1}$)
−10 to 0	5.8 ± 1.2 (5)
0 to 1	102.7 ± 18.4 (5)
1 to 10	32.2 ± 10.8 (5)

Glucose was added at time zero at a concentration of 10 mM to a 5% suspension of cells at 37° C. The pH was maintained constant at 7.4 by regulated addition of 0.02 N NaOH. Standard deviations and the number of experiments are indicated.

The results to this point confirmed the previously observed stimulation of acidification by glucose, but they also showed that continued acidification could produce a proton concentration gradient from medium to cell. We next quantified H^+ efflux from these cells.

Measurement of H^+ Efflux at Constant Medium pH

We used the pH-stat method, as described in Materials and Methods, to quantify the rate of H^+ exit from the cells at a constant medium pH of 7.4. The cell suspension was again 5% (v/v) in the same weakly phosphate-buffered solution used for the acidification experiments above. The results of five such experi-

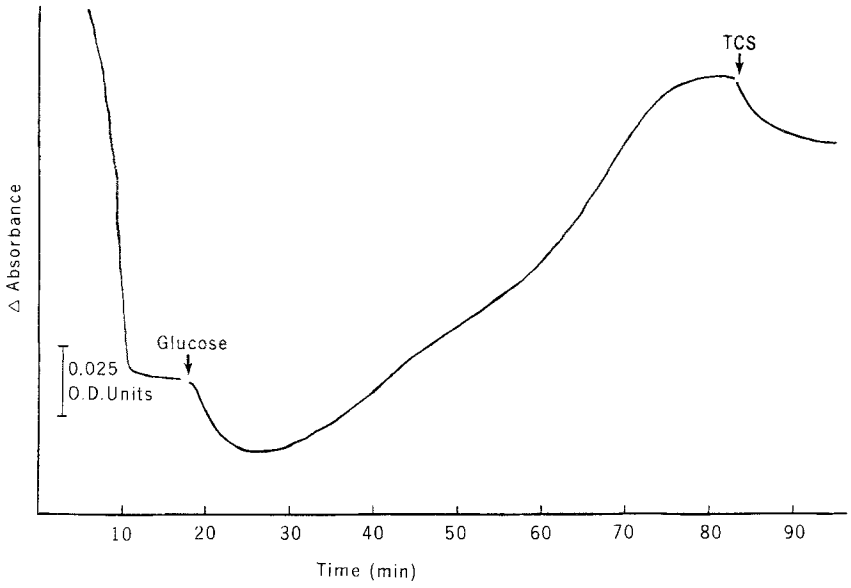

Fig. 2. Changes in H^+ distribution across the cell membrane. The relative absorbance of acridine orange (added at 15 μM) indicates the pH of the cells relative to the medium. For example, decreasing differential absorbance (490 nm relative to 530 nm) indicates acridine uptake by the cells produced by acidification of the cellular compartment relative to the medium. Times of addition of 10 mM glucose and 5 μM TCS are indicated

ments are shown in Table 1. Before glucose addition the basal rate of acidification compared quite favorably with the value of about 8–10 μM min^{-1} g^{-1} reported previously by Heinz et al. (1977). Addition of 10 mM glucose produced a dramatic initial 18-fold increase in H^+ secretion lasting 30–60 sec which then decreased to a steady rate about fivefold greater than the basal rate. The time course of the transition from rapid to slower H^+ extrusion corresponded well to that observed for medium acidification (Fig. 1). In additional experiments which are not shown, the rate of medium acidification was measured in cell suspensions after addition of either 10 μM nigericin or 0.1 μM valinomycin. Five min after adding 10 mM glucose in these experiments, the rate of acidification was 22–32 μmol min^{-1} g^{-1} as observed previously (Table 1). Addition of nigericin at this point increased H^+ efflux to 98–106 μmol min^{-1} g^{-1} (2 experiments) and valinomycin increased H^+ efflux to 66–79 μmol min^{-1} g^{-1} (2 experiments).

In order to support the conclusion from the acidification experiments with TCS (Fig. 1) that an inward-directed H^+ gradient is developed by glucose-induced acidification, the relative changes in intracellular pH were examined using acridine orange.

Acridine Orange Experiments

Acridine orange was used to indicate the pH of the cells relative to that of the medium. This dye was added at a concentration of 15 μM to a 5% cell suspension in well-buffered (10 mM phosphate; pH 7.4), low Na^+ medium. The absorbance of acridine orange at 490 nm relative to the reference wavelength (530 nm) was observed to fall after its addition due to acridine orange equilibration with the cell interior. After a stable absorbance difference was obtained, the addition of 10 mM glucose resulted in a further dramatic decrease in absorbance as shown in Fig. 2. This result indicated that the pH of the intracellular compartment acidified relative to the medium. However, within 10 min after glucose addition, acridine orange began to exit from the cell resulting in an increasing absorbance which exceeded the initial absorbance after about 20 min. As shown in Fig. 2, subsequent addition of 5 μM TCS reversed the direction of acridine orange movement, showing that TCS allowed proton movement into the cell down an electrochemical potential gradient.

DOCC Experiments

In order to assess the nature of the apparent extrusion of protons against a concentration gradient, we followed changes in the transmembrane voltage using DOCC. Figs. 3 and 4 show the differential absorbance of DOCC at 580 nm relative to a reference wavelength of 630 nm. After adding 10 μM DOCC to a 5% suspension of cells in the low Na^+ solution (10 mM phosphate, pH 7.4), there was a rapid decrease in absorbance due to accumulation and quenching of the positively charged dye in the relatively electronegative cell interior (Waggoner et al., 1977; Rabon et al., 1978). From previous unpublished experiments, which employed the null-point method of Philo and Eddy (1978) for calibrating the transmembrane voltage using valinomycin with various external K^+ concentrations, we determined that the initial resting transmembrane voltage was -32 ± 3 mV. Addition of 10 mM glucose to the same suspension resulted in a dramatic and immediate hyperpolarization evidenced by the dye movement into the cells (Fig. 3). We cannot quan-

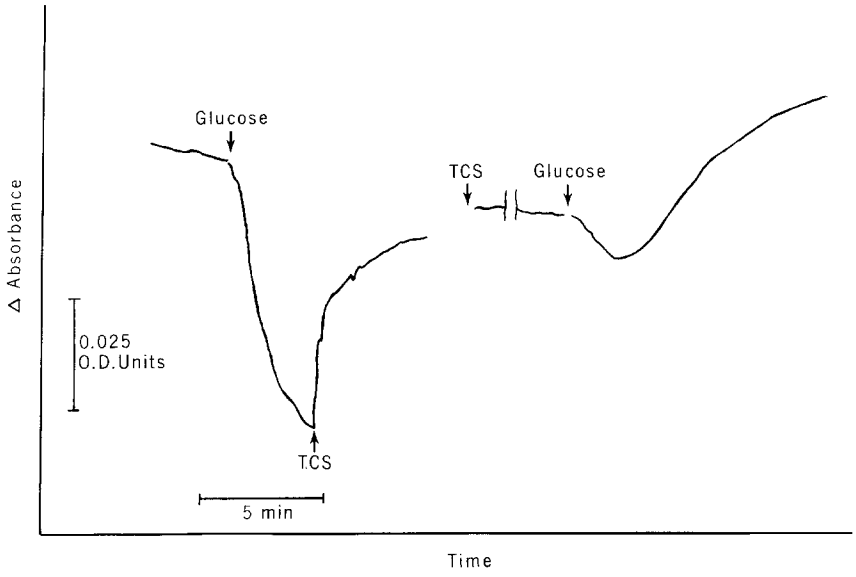

Fig. 3. Changes in membrane voltage associated with glucose addition in the presence and absence of TCS. Differential absorbance due to DOCC (absorbance at 580 nm relative to 630 nm) is shown subsequent to equilibration of dye with the cells. Decreased differential absorbance reflects hyperpolarization due to a shift of the positively charged dye into the cell with a resulting decrease in absorbance at 580 nm. Five μM TCS was added either 2 min after addition of 10 mM glucose (left-hand plot), or 10 min before (right-hand plot)

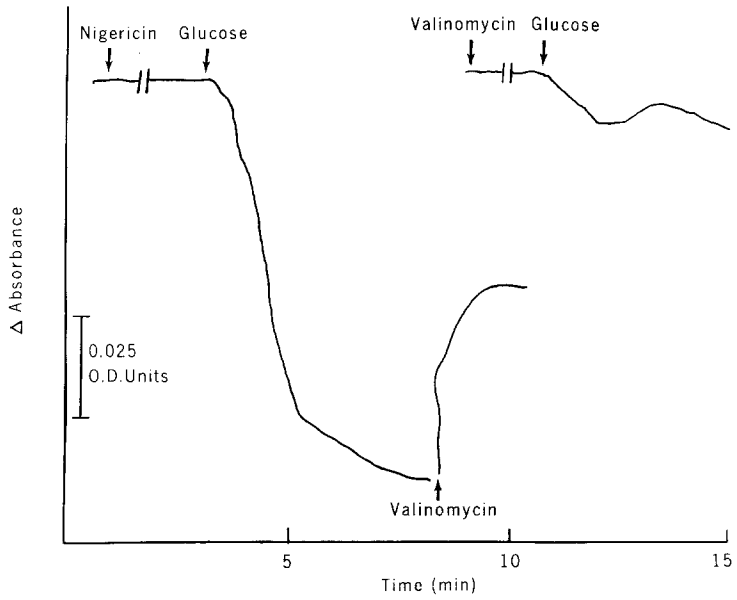

Fig. 4. Effects of nigericin and valinomycin on glucose-induced hyperpolarization. Ten μM nigericin was added 10 min prior to addition of 10 mM glucose. Valinomycin (0.1 μM) was added either 5 min after glucose (left-hand plot), or 5 min prior to glucose (right-hand plot)

tify the voltage precisely under these circumstances, but from previous valinomycin-K^+ null-point observations we would expect the potential to be more negative than −100 mV. As would be expected, the imposition of a highly conductive electrical shunt produced by the addition of 5 μM TCS rapidly and markedly reversed the hyperpolarization produced by glucose (Fig. 3, left) Prior addition of TCS (Fig. 3, right) prevented the hyperpolarization upon glucose addition.

Addition of 10 μM nigericin 5 min prior to D-glucose addition had no effect on the hyperpolarization (Fig. 4), but addition of ~0.1 μM valinomycin to the suspension in the presence of 20 mM extracellular K^+ produced a depolarization presumably to the existing K^+ equilibrium potential of about −50 mV, indicating that the membrane voltage had been considerably more negative than −50 mV after glucose addition. As shown in the right-hand trace of Fig. 4, when 0.1 μM valinomycin was added 2 min prior to addition of 10 mM glucose, the hyperpolarization was largely prevented. The remaining hyperpolarization most likely represents the K^+ equilibrium voltage which exceeded the resting membrane voltage (*see above*). The glucose-induced hyperpolarization was observed to persist for far longer than 30 min, i.e. for at least as long as was required to establish the inward-directed H^+ concentration gradient (*see* Figs. 1 and 2).

Addition of a 10-mM concentration of 2-deoxy-D-glucose, L-glucose, mannitol, lactate, pyruvate or as-

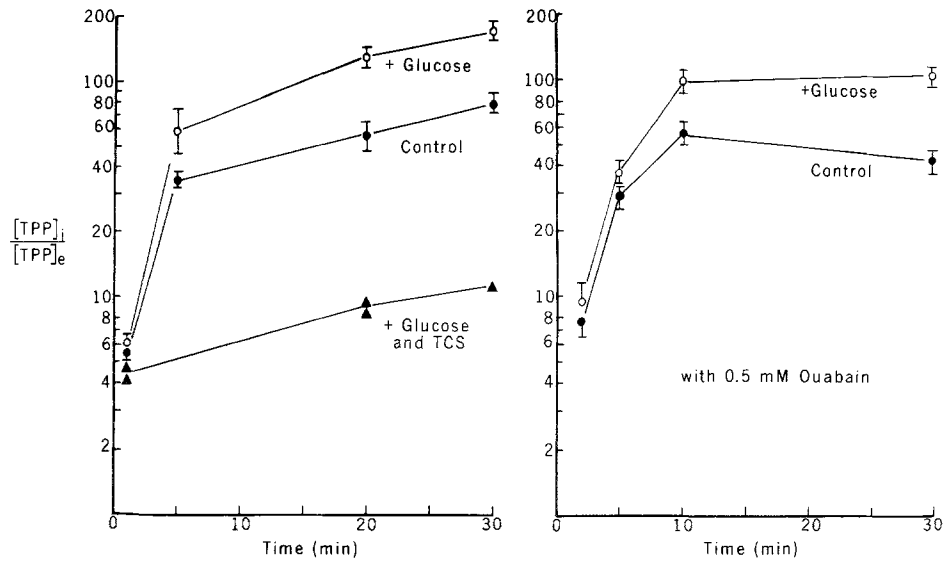

Fig. 5. Effect of glucose on the distribution of TPP^+. *Left hand plot*: The H-TPP^+ distribution ratio is plotted as a function of time either after addition of normal medium (control), after addition of 10 mM glucose alone, or after addition of 10 mM glucose + 5 μM TCS (results of 4 experiments. *Right hand plot:* Same experiment performed in the presence of 0.5 mM ouabain in the medium (results of three experiments)

corbate all produced less than a 0.004 O.D. change in the differential absorbance due to DOCC, whereas 10 mM D-glucose decreased differential absorbance by 0.054 ± 0.017 O.D. units. Thus, as in the case of acidification, the hyperpolarization appeared to be induced specifically by glucose.

In additional experiments which are not shown, addition of 0.1 mM ouabain had no effect on the glucose-induced hyperpolarization, and nor did 50–100 μM vanadate [an inhibitor of (Na+K)-ATPase] when added alone or in combination with 0.1 mM ouabain 10 min prior to glucose. These results indicate that the hyperpolarization was not the consequence of increased activity of an electrogenic (Na+K)-ATPase or of a diffusion potential (*see below*).

Distribution of Tetraphenylphosphonium (TPP^+)

The distribution of TPP^+ was used as an additional means of assessing changes in membrane voltage with glucose. TPP^+ is a lipid-permeant cation which has been shown to equilibrate within 10 min in accordance with membrane voltages in the Ehrlich ascites tumor cell (Heinz et al., 1975). As observed by Heinz et al. (1975) TPP^+ accumulates to a distribution ratio in excess of 60 in the absence of substrate. This ratio exceeds that expected for the presumed plasma membrane voltage of about −30 mV; however, the additional accumulation probably represents mitochondrial uptake (Heinz et al., 1975). Of importance to the present studies, when 10 mM glucose was added with ^{3}H-TPP^+ the distribution ratio of the latter increased to greater than 160 as shown in Fig. 5 (left side). (It should be noted that this is a logarithmic plot of the TPP^+ distribution ratio.) Addition of 5 μM TCS simultaneous with glucose and ^{3}H-TPP yielded TPP^+ distribution ratio of only 9–11. The paired difference between ^{3}H-TPP^+ distribution ratios obtained from the same cell suspensions at 20–30 min was 80 ± 13 ($p < 0.001$). According to the calibration curve of Heinz et al. (1975), this increase in TPP^+ distribution ratio would represent a hyperpolarization of the negative membrane voltage by more than 100 mV. The same experiments were conducted in the presence of 0.5 mM ouabain and the results are shown on the right-hand plot of Fig. 5. Again, D-glucose produced a marked increase in the TPP^+ distribution ratio.

Effect of Inhibitors of Lactate Transport

Spencer and Lehninger (1976) have reported that lactate transport in Ehrlich ascites tumor cells occurs as a passive, electroneutral movement of the anion together with H^+ or in exchange for OH^-. In order to investigate whether this mechanism might also be involved in the active, electrogenic H^+ efflux demonstrated by the above studies, we examined the effect of inhibitors of the lactate carrier on the rate of acidification and hyperpolarization produced by glucose. Spencer and Lehninger (1978) showed that α-cyano-4-hydroxycinnamate and α-cyano-3-hydroxycinnamate are competitive inhibitors of the lactate carrier with K_i values of 0.5 and 2 mM, respectively. As shown in the upper portion of Table 2, 2.5 mM α-cyano-4-hydroxycinnamic acid did inhibit both the rapid and

Table 2. Effect of lactate transport-inhibitors on acidification and hyperpolarization produced by glucose

A. Acidification rate (n=5)			
Condition	Control	+2.5 mM α-cyano-4-hydroxy-cinnamic acid	*p*
	pH units/min		
Before glucose	0.02±0.00	0.02±0.00	N.S.
0–30 sec after 10 mM glucose	0.39±0.05	0.09±0.01	$p<0.05$
60–180 sec after 10 mM glucose	0.07±0.00	0.04±0.00	$p<0.001$

B. TPP^+ distribution ratio (n=3)	
Additions to medium	10-min ratio of intra- to extracellular TPP^+ concentrations
Control	47.1±1.7
10 mM glucose	87.3±7.4
10 mM glucose + 2.5 mM α-cyano-4-hydroxycinnamic acid	88.4±7.5
10 mM glucose + 5 mM α-cyano-3-hydroxy-cinnamic acid	84.9±8.0

Acidification experiments were conducted as described previously for the results shown in Fig. 1. TPP^+ distribution ratios were measured 10 min after addition of ^{3}H-TPP^+ with or without 10 mM glucose. In both sets of experiments, the indicated inhibitors were added 10 min before glucose.

the slow phase of glucose-induced acidification as expected. However, the rate of acidification of the medium was still significantly greater in the presence of glucose than in the absence. The medium pH in the presence of glucose fell to 6.5 within 20 min, and at this point addition of 5 μM TCS also resulted in a transient alkalinization of the medium as had been observed in control experiments (Fig. 1). Similar results were obtained when 5.0 mM α-cyano-3-hydroxycinnamic acid was used as the inhibitor. The observation show that in spite of a reduced rate of acidification, the glucose-induced H^+ movement still results in an inward-directed proton gradient.

In contrast to their effects on acidification, the same two inhibitors had no effect on the glucose-induced hyperpolarization. As shown in the lower half of Table 2, the distribution ratio of TPP^+ after a 10-min incubation in the presence of glucose was unaltered in the presence of the inhibitors. Similarly, the inhibitors had no significant effect on the DOCC differential absorbance change produced by 10 mM glucose. In these experiments, cells were incubated for 10 min with DOCC and with or without inhibitors. In control cells 10 mM glucose resulted in a 0.075±0.015 (n=4) O.D. unit decrease in the differential absorption within 30 sec after addition. In the same experiments glucose addition after prior addition of 2 mM α-cyano-4-hydroxycinnamate or 5 mM α-cyano-3-hydroxycinnamate gave the same absorbance changes: 0.077±0.003 (n=3) and 0.092 (n=1), respectively.

Discussion

We interpret the results presented above to suggest the presence of an active electrogenic proton pump which is activated by glycolysis, in Ehrlich ascites tumor cell membranes. We discuss below our rationale for this conclusion on the basis of the various experimental results. As an aid to following these arguments, Fig. 6 illustrates schematically what we propose to be the relative H^+ distribution and transmembrane voltage in the following three situations: A) in the absence of exogenous substrate, B) within the first minute after glucose addition, and C) when the external pH has fallen to less than approximately 6.5 in the presence of glucose. The experimental situation would be the same as in Fig. 1 at −1, +1, and +5 min, respectively. The sizes of the letter "H" illustrate the relative H^+ concentrations. The larger minus signs in Figs. 6*B* and 6*C* depict the hyperpolarization observed with glucose addition.

In the absence of exogenous substrates, Geck et al. (1978) have shown that the cytoplasmic H^+ concentration is actually slightly less than the extracellular concentration. Since the transmembrane voltage is inside-negative in this situation, they proposed that the non-equilibrium H^+ distribution could be produced by an active proton efflux mechanism. Our experiments provide no new information concerning this possibility under these conditions, and as shown in Fig. 6*A* we assume the intra-and extracellular pH to be nearly equal, and the membrane voltage to be about −40 mV. Under these control conditions we observed that the medium slowly became more acidic. From pH-stat measurements, the H^+ efflux from the cells was 5.8 μmol min^{-1} g^{-1}, which is almost identical to that observed previously by Heinz et al. (1977). This basal rate of acidification was not inhibited by CN^- indicating that that it is not dependent on mitochondrial respiration. We do not depict any H^+ pump mechanism for this process in Fig. 6*A* because, if it exists, it operates at a negligible rate compared to that observed after glucose addition. The substrate responsible for the bassal acidification rate has been

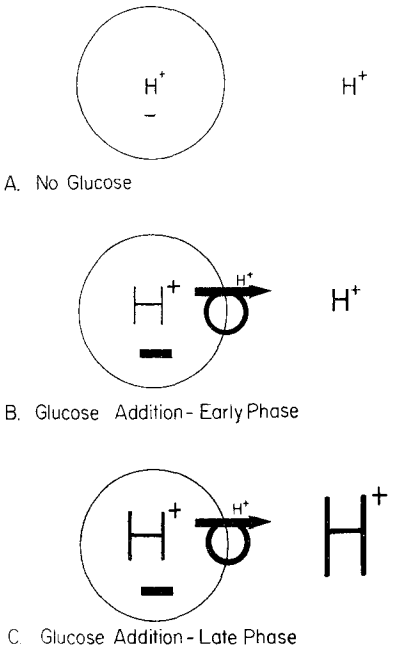

Fig. 6. Schematic representation of the H^+ distribution and membrane voltage in the Ehrlich ascites tumor cell. (*A*) Before the addition of glucose and in the absence of other exogenous substrates. (*B*) 30–60 sec after the addition of 10 mM glucose. (*C*) Two or more min after glucose addition during the slow phase of acidification. The sizes of the symbol H^+ represent relative H^+ concentrations. The size of the minus sign illustrates the degree of hyperpolarization. The circle and arrow represent the electrogenic H^+ pump mechanism

identified and the process has not been examined further in these studies.

Fig. 6*B* depicts the situation shortly after the addition of 10 mM D-glucose to the medium. Rapid glycolysis, especially in the first minute after glucose addition, exceeds the mitochondrial respiratory capacity and results in lactic acid formation and intracellular acidification (Racker, 1956; Poole, 1967; Thomas et al., 1979). Under these conditions, there is concomitant acidification of the medium (Fig. 1); however, due to rapid lactic acid production the cytoplasm becomes more acid relative to the extracellular medium as evidenced by the shift of acridine orange into the cells (Fig. 2). Immediately upon glucose addition, there is also a strong hyperpolarization of the transmembrane voltage, which is evidenced by the rapid fall in DOCC absorbance (Figs. 3 and 4) and by the increased TPP^+ distribution ratio in the presence of glucose (Fig. 5). We interpret this hyperpolarization to be due to activation of an electrogenic H^+ transport process. This conclusion is supported by the observation that the protonophore TCS immediately reverses the hyperpolarization when added after glucose, and prevents it if added before (Figs. 3 and 5). Since neither ouabain (Fig. 5) nor vanadate prevented the hyperpolarization, it cannot be attributed to activation of an electrogenic (Na+K)-ATPase.

As shown in Fig. 6*C*, after about 1 min when the rate of acidification has declined (Fig. 1), presumably due to decreased glycolysis, continued active H^+ transport produces an inwardly directed H^+ electrochemical potential gradient. This conclusion is supported by the finding that the addition of TCS at this point results in a transient alkalinization of the medium due to proton backflux into the cells (Fig. 1). Because TCS also produces depolarization of the membrane voltage when added at this time (Figs. 4 and 5), the passive backflux indicates that the H^+ electrochemical potential gradient just before TCS addition was even greater and directed inward. The inward direction of the H^+ electrochemical potential gradient in the later phase is also demonstrated by the uptake of acridine orange after TCS addition (Fig. 2). These findings demonstrate that the H^+ efflux in the later phase of glucose-induced acidification occurs against an electrochemical potential gradient. Thus, in the broadest sense, the H^+ efflux must be considered an active transport process, although whether it is directly linked to a chemical reaction such as ATP hydrolysis or whether it is coupled to the movement of another solute remains to be determined.

As would be expected for an electrogenic active transport mechanism, prior addition of valinomycin blunts the hyperpolarization produced by glucose (Fig. 4), and by reversing the glucose-induced hyperpolarization valinomycin increases the rate of proton efflux. On the other hand, as expected, the neutral-exchange ionophore nigericin has no effect on the hyperpolarization induced by glucose (Fig. 4) but increases the H^+ efflux (pH-stat) by providing another H^+ exit route. The results with nigericin also argue against the possibility that the glucose-induced hyperpolarization is produced by a diffusion potential resulting from ion redistribution. Nigericin would be expected to alter diffusion potentials by producing rapid changes in ion concentration gradients but, as a neutral ionophore, it should have no effect on an electrogenic potential.

It has been proposed previously that acidification of the medium during glycolysis occurs largely as the consequence of mediated efflux of undissociated lactic acid (Poole, 1967; Spencer & Lehninger, 1976; Thomas et al., 1979). The present results do not establish what fraction if any of the H^+ efflux engendered by glycolysis occurs by this mechanism. The mechanism of lactic acid efflux proposed by Spencer and Lehninger (1976) involved an electroneutral exit of

H^+ and lactate anion via a common facillitating site. Furthermore, they proposed this exit to be purely dissipative, i.e. lactic acid efflux would be driven by its own concentration gradient. The above results show that within 5–10 min after the addition of D-glucose, the extracellular H^+ concentration exceeds the intracellular. During the development of this H^+ concentration gradient the membrane is dramatically hyperpolarized. Comparison of the decrease in DOCC differential absorbance to that produced by K^+ distributions in the presence of valinomycin, and the magnitude of the change in the distribution ratio of TPP^+, suggest that the membrane voltage is well in excess of -100 mV in the presence of glucose. Therefore, H^+ effluxes against an extremely high electrochemical potential gradient. This apparently active efflux could be coupled to the efflux of lactate down its electrochemical potential gradient, but it would not explain the hyperpolarization if the process were electrically neutral. It is possible that under these circumstances the co-transport mechanism proposed by Spencer and Lehninger (1976) could operate electrogenically, i.e. with a H^+:lactate stoichiometry in excess of 1:1. This possibility is contradicted, however, by the observation that inhibitors of the lactate carrier system, α-cyano-4-hydroxycinnamate and α-cyano-3-hydroxycinnamate (Spencer & Lehninger, 1976) have no effect on the hyperpolarization observed after glucose addition as assessed both by the change in DOCC absorbance and the TPP^+ distribution ratio. These inhibitors did inhibit the rate of acidification after glucose addition as would be expected from the results of Spencer and Lehninger (1976). But since an inward-directed H^+ electrochemical potential gradient was still established and the magnitude of the hyperpolarization was unaltered by the inhibitors, we presume that the postulated electrogenic H^+ transport mechanism is not associated directly with the lactate carrier mechanism.

With respect to the hyperpolarization produced by glucose, the above results link it only temporally with H^+ efflux. However, the hyperpolarization does not appear to be due to a diffusion potential since it is unaffected by the presence of nigericin. Furthermore, the hyperpolarization has an immediate onset and rises rapidly to a maximum well before the acidification rate reaches a plateau, and it is immediately abolished or reduced by TCS and valinomycin. All of these results point to an electrogenic pump mechanism activated by glucose or glycolysis. Our preference for an electrogenic H^+ pump is based on the fact that active H^+ extrusion occurs during the hyperpolarization and that the active H^+ efflux is accelerated by depolarizing the membrane voltage with TCS or valinomycin. Our preference is also conditioned by previously described electrogenic transport mechanisms in other cells. To our knowledge these include active transport mechanisms only for Na^+-K^+, Ca^{++} and K^+. However, there is no evidence for Ca^{++} involvement in the glucose-induced hyperpolarization because it is absent from the bathing solution in these experiments. And the contribution of an electrogenic Na^+-K^+ pump to the hyperpolarization is ruled out by the lack of effect of ouabain or vanadate.

It is possible that another electrogenic process may be involved. For example, Geck et al. (1980) have demonstrated a cotransport mechanism for Na^+-K^+-$2Cl^-$, which is inhibited by furosemide, in the Ehrlich cell. A similar furosemide-sensitive cotransport mechanism has been described in duck red cells, but in this system it appears that stoichiometric requirements can change depending on the tonicity of the medium and other factors (Schmidt & McManus, 1977). Although both mechanisms, as presently described, operate in a passive and electroneutral fashion, it is conceivable that either could be activated to produce electrogenic H^+ active transport.

Also, our data do not establish conclusively whether the hyperpolarization occurs across the plasma membrane or the membrane of a cellular organelle. Because the distributions of both DOCC and TPP^+ are volume- as well as voltage-sensitive, if an organelle were presumed to account for the observed hyperpolarization and if that organelle occupied even 10% of the total cell volume, then the hyperpolarization observed would correspond to a change of at least 180 mV across the organelle membrane. In the case of mitochondria, the membrane voltage may be close to -160 mV before addition of glucose, and the final voltage would be predicted to be -340 mV which seems unlikely. Hence, if the hyperpolarization does not occur across plasma or mitochondrial membranes, another organelle must be involved, which also seems unlikely.

The reason why glucose addition activates the pump mechanism remains obscure, although the process is clearly associated with glycolysis. This H^+ efflux and hyperpolarization are unaffected by CN^- or by amytal. Furthermore, no stimulation of acidification or hyperpolarization is produced by other substrates or by the nonmetabolizable, transported sugar 2-deoxy-D-glucose. It is also interesting that the time course of acidification follows that expected for glycolysis after glucose addition. As reported previously (Chance & Hess, 1956), after glucose addition glycolysis is rapid but slows after ~1 min. The same is observed for the time course of acidification (Fig. 1). Racker (1956) has suggested that glycolysis is limited as a consequence of the concomitant fall in intracellular pH. Since the pH-stat experiments also demon-

strated the same biphasic rate or H^+ efflux at a constant extracellular pH, these results show that, if pH is the inhibitor of the glycolytic rate, it must be the pH of the intracellular compartment. A limiting medium pH of 5.5 is reached only due to the concomitant reduction in intracellular pH; i.e., the pump can only maintain a finite electrochemical potential difference for H^+ between intra- and extracellular compartments. When the extracellular pH is maintained at 7.4 by the pH-stat method, we have observed that the H^+ efflux remains high indefinitely.

In summary, we interpret our results to provide evidence for the presence of an active, electrogenic H^+ efflux mechanism which is activated by glycolysis, and which appears to be limited in its operation by the rate of glucose entry and/or glycolysis. Several important questions remain for future investigations. We do not know what activates the proposed proton pump. It could be activated by the increased intracellular H^+ concentration and/or ATP. Also, we have not identified the nature of the energy source for the active transport. It could be driven by ATP hydrolysis, by a redox mechanism, or by coupling to the flow of another solute, although the latter possibility appears remote due to the electrogenic nature of the process. Finally, it will be important to identify conclusively the site of hyperpolarization since only indirect arguments thus far support the plasma membrane as the location of the electrogenic pump.

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