J. Membrane Biol. 5, 261–276 (1971)


# Effects of Valinomycin, Ouabain, and Potassium on Glycolysis and Intracellular pH of Ehrlich Ascites Tumor Cells*

DORIS T. POOLE, THOMAS C. BUTLER**, and MARY E. WILLIAMS

Center for Research in Pharmacology and Toxicology,
University of North Carolina School of Medicine,
Chapel Hill, North Carolina 27514

Received 5 February 1971

*Summary.* Both valinomycin and ouabain block reaccumulation of $K^+$ by Ehrlich ascites tumor cells depleted of $K^+$ and cause loss of $K^+$ from high-$K^+$ cells. Glucose largely reverses the effect of valinomycin and to a lesser extent that of ouabain.

In cells depleted of $K^+$, glucose utilization and lactate production are impaired. Neither extracellular pH ($pH_e$) nor intracellular pH ($pH_i$) falls to the extent seen in non-depleted glycolyzing cells. Addition of $K^+$ to depleted cells reverses these effects. Valinomycin increases glycolysis in $K^+$-depleted cells but to a greater extent in non-depleted or $K^+$-repleted cells. The increase in lactate production caused by valinomycin is accompanied by a correspondingly greater fall in $pH_e$ and $pH_i$. Valinomycin, unlike other uncoupling agents, does not abolish the pH gradient across the plasma membrane. Increased utilization of glucose resulting from addition of $K^+$ to $K^+$-depleted cells or addition of valinomycin either to depleted or non-depleted cells can be entirely accounted for by increased lactate production. Ouabain blocks the stimulatory effect of added $K^+$ on $K^+$-depleted cells and has an inhibitory effect on glycolysis in non-depleted cells. It does not obliterate the difference in glycolytic activity between $K^+$-depleted and non-depleted cells. Ouabain does not completely block the effect of valinomycin in augmenting glycolysis in depleted or non-depleted cells. Increased accumulation of glycolytic intermediates, particularly dihydroxyacetone phosphate, is found in glycolyzing $K^+$-depleted cells. The most marked accumulation was found in ouabain-treated $K^+$-deficient cells.

The cyclododecadepsipeptide ionophorous antibiotic, valinomycin, was first shown to be an uncoupler of oxidative phosphorylation (McMurray & Begg, 1959). Moore and Pressman (1964) reported that valinomycin causes accumulation of $K^+$ by mitochondria with ejection of $H^+$. Subsequently many studies demonstrated an effect of valinomycin in increasing $K^+$ per-

* This investigation was supported by U.S. Public Health Service grant no. GM 13606 from the National Institute of General Medical Sciences and by grant no. P-603 from the American Cancer Society.

** PHS Research Career Program Award 4 K06 GM 19429 from the National Institute of General Medical Sciences.

meability of membranes of mitochondria, bacterial chromatophores, and erythrocytes, and of artificial lipid membranes and bulk organic phases. The effect of valinomycin in enhancing the permeability of membranes to $K^+$ has been attributed to the formation of a lipid-soluble $K^+$-valinomycin complex. Valinomycin shows a marked selectivity for complexing with $K^+$ in comparison with $Na^+$.

Schatzmann (1953) reported that *k*-strophanthin and digitoxin and their aglycones inhibit the active transport of $Na^+$ and $K^+$ through erythrocytic membranes. Post, Merritt, Kinsolving, and Albright (1960) identified in the membrane of the erythrocyte an ATPase activated by $Na^+$ plus $K^+$. Ouabain exerted an inhibitory action on this enzyme similar to that on the ion pumping mechanism in the membrane. There have been numerous subsequent studies of the effects of cardiac glycosides on the $(Na^+ + K^+)$-dependent ATPase and on the transport of $Na^+$ and $K^+$ across membranes of many types of cells. Because of its relatively high solubility in water, ouabain has been the cardiac glycoside most often used in biochemical studies of alkali ion transport.

There have been several studies of the effects of valinomycin or ouabain or both on tumor cells. The two drugs have sometimes been used together in an effort to separate the effects of plasma membrane and mitochondrial membrane $K^+$ transport.

Maizels, Remington, and Truscoe (1958*a*, *b*) found that when Ehrlich ascites tumor cells were cooled, $Na^+$ and $K^+$ flowed with the concentration gradients with loss of $K^+$ from the cell and entry of $Na^+$ into the cell. On warming in the presence of $K^+$, the flow was reversed. Cells washed in a cold $K^+$-free medium containing $Na^+$ lost nearly all their $K^+$ and gained $Na^+$. When transferred to a warm $K^+$-containing medium, there was rapid entry of $K^+$ into the cell and loss of $Na^+$. $K^+$-depletion caused a marked decrease in glycolysis and a small decrease in respiration. They also reported that ouabain and digoxin retarded the efflux of $Na^+$ from cells enriched in $Na^+$ and depleted of $K^+$ when they were returned to the $K^+$-containing medium. Bittner and Heinz (1963) found that ouabain caused loss of $K^+$ from Ehrlich ascites tumor cells in concentrations above $10^{-4}$ M but did not affect oxygen consumption at a concentration of $10^{-3}$ M.

Effects of valinomycin on light scattering in the intact Ehrlich ascites tumor cell were first reported by Wenner, Harris, and Pressman (1966). These authors (1967) in an experiment with glycolyzing Ehrlich-Lettré tumor cells found a continuous loss of $K^+$ from the cells with a sharp, transient increase on addition of valinomycin. Valinomycin caused increases in the rates of oxygen consumption and acid production. Results were similar in

MClM rhabdomyosarcoma ascites tumor cells except that there was no movement of $K^+$. Gordon, Nordenbrand, and Ernster (1967) found that valinomycin increased oxygen consumption and acid production in Ehrlich ascites tumor cells metabolizing endogenous substrates. In cells depleted of $K^+$, this effect of valinomycin was lacking and could be restored by addition of $K^+$ to the medium. In the presence of ouabain, addition to $K^+$-depleted cells of either $K^+$ or valinomycin alone was without effect on oxygen uptake and acid production, but addition of both $K^+$ and valinomycin had a stimulating effect. Levinson (1967) studied the uptake of $K^+$ and elimination of $Na^+$ by Ehrlich-Lettré ascites tumor cells that had been depleted of $K^+$ and then incubated in a $K^+$-containing medium. The transport of these ions was completely blocked by ouabain. Valinomycin inhibited ion movement concomitantly with ATP depletion. Addition of glucose released the inhibition of ion movement as the level of ATP was restored. Levinson and Hempling (1967) found that, during repletion of $K^+$-depleted Ehrlich tumor cells, the transport of $Na^+$ and $K^+$ is not necessarily coupled in a 1:1 ratio. Addition of $K^+$ to the external medium stimulated respiration of $K^+$-depleted cells, and this stimulation could be inhibited with ouabain. Gordon and de Hartog (1968) reported that the rate of acid production in $K^+$-depleted Ehrlich ascites tumor cells metabolizing glucose was low and was unaffected by valinomycin. Acid production was increased by addition of $K^+$ to the incubation medium and to a greater extent by addition of both $K^+$ and valinomycin. Ouabain was without effect on glucose utilization and lactate production in $K^+$-depleted cells. In $K^+$-containing cells, ouabain diminished glucose utilization but did not block the stimulation of glycolysis by valinomycin.

None of these studies of glycolysis of tumor cells as affected by valinomycin, ouabain, or $K^+$ has included measurements of glycolytic intermediates or intracellular pH ($pH_i$). It seemed that these measurements might furnish information pertinent to the elucidation of a number of questions concerning the actions of valinomycin, ouabain, $K^+$, and combinations of the three in glycolysis. It was of interest to learn if valinomycin would have the same effect on the pH gradient across the plasma membrane of glycolyzing cells as had been found for other uncouplers (Poole, 1968). If the $K^+$–$H^+$ exchange observed with valinomycin in isolated mitochondria should occur in the intact cell, this might change $pH_i$ in a way not seen with other uncouplers. A study of glycolytic intermediates and $pH_i$ in glycolysis inhibited by $K^+$-depletion was desirable for comparison with the patterns produced by drugs that inhibit the glycolytic enzymes (Poole & Butler, 1969). We also wished to study possible correlations of intra-

cellular $K^+$ with the metabolic alterations produced by the various treatments.

The present studies of the effects of valinomycin, ouabain, and $K^+$ depletion and repletion have been carried out with the Ehrlich ascites tumor cell. The measurements of $pH_i$ were made by a method based on the distribution between intracellular and extracellular water of the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO). The use of DMO for this purpose was first proposed by Waddell and Butler (1959), and a method employing DMO-$^{14}$C adapted to studies of cell suspensions *in vitro* has been applied in several previous investigations of $pH_i$ in the Ehrlich ascites tumor cell (Poole, Butler, & Waddell, 1964; Poole, 1967, 1968; Poole & Butler, 1969).

## Materials and Methods

Valinomycin was purchased from Calbiochem. Ouabain was obtained from Sigma Chemical Co. The sources of other chemicals and enzymes were previously described (Poole & Butler, 1969).

### *Preparation of Cells*

The Ehrlich ascites tumor cells were grown and harvested as has been described (Poole *et al.*, 1964). Three procedures for washing the cells were followed. In one, the Krebs-Ringer buffer used had the following composition: 0.147 M NaCl, 0.006 M KCl, 0.001 M $MgSO_4$, 0.021 M $Na_2HPO_4$, and 0.004 M $NaH_2PO_4$. It had a pH of 7.35 at 37 °C. The cells were washed and centrifuged in the cold six times at 30-min intervals. In the second procedure, the cells were depleted of $K^+$ by washing in the same manner with a buffer differing from that described above in that the KCl was replaced by an equivalent amount of NaCl. In another series of experiments, in order to minimize loss of $K^+$, the cells were washed rapidly four times in the $K^+$-containing buffer.

### *Incubations*

The cells were suspended in the same buffer in which they had been washed. The concentrations of the suspensions are given in the figure legends. The suspensions were shaken in beakers at 37 °C under an atmosphere of $O_2$. In those experiments in which $pH_i$ was to be measured, DMO-$^{14}$C to a concentration of 0.05 μC (6.5 μg) per ml was added to the suspensions 15 min before further additions were made. Additions were made and samples were taken as described in the figures and their legends. Additions were made in the following forms: 110 mM glucose in buffer, 0.1 ml per ml suspension; 0.25 M KCl in buffer, 0.02 ml per ml suspension; $10^{-3}$ M valinomycin in ethanol, 0.01 ml per ml suspension; $10^{-2}$ M ouabain in buffer, 0.1 ml per ml suspension. In experiments in which comparisons were to be made with and without valinomycin, ethanol in the same volume was added to the valinomycin-free suspensions.

### *Calculation of $pH_i$*

After incubation, the suspensions were centrifuged as previously described (Poole, 1967). In these experiments, direct determination of the extracellular water in the cellular layer by means of inulin-carboxyl-$^{14}$C was omitted. The extracellular water of the

cellular layer was assumed to be 30% of its total water. In large numbers of experiments previously carried out under identical conditions, the extracellular space was found to deviate little from this value. Otherwise, measurement of $pH_e$ and DMO and calculation of $pH_i$ were carried out as previously described (Poole, 1967).

### *Analytical Methods*

The enzymatic methods used for determination of glucose and lactate in the supernatant fluid and of fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate in total cell suspensions have been cited in an earlier publication (Poole & Butler, 1969).

When cellular $K^+$ was to be determined, a sample of about 100 mg of the centrifuged cellular pellet was digested overnight at room temperature in 1.0 ml of 0.1 N $HNO_3$. This digest was centrifuged and $K^+$ was determined in the supernatant by flame photometry. Calculation of cellular $K^+$ in terms of mequiv/liter of cell water was made by correcting for the $K^+$ trapped in the extracellular space. This correction was made from the $K^+$ concentration measured in the suspension supernatant, the assumption being made that the extracellular water of the cellular pellet is 30% of its total water.

### *Calculation of Glucose Utilization and Lactate Production*

The total glucose and lactate present at the end of an incubation were calculated from the concentrations of those substances in the supernatant fluid on the assumptions that all glucose is extracellular and that lactate is present in equal concentrations intracellularly and extracellularly. The packed cell volume for each suspension was determined by the micro-hematocrit method. Glucose utilization and lactate production were calculated in terms of μmoles/ml of packed cell volume.

## Results

### *Effects of Valinomycin and Ouabain on Intracellular $K^+$ in $K^+$-Depleted Cells and Non-Depleted Cells*

Our procedure in which the cells were washed six times with a $K^+$-free buffer in the cold resulted in intracellular $K^+$ concentrations ranging from 3 to 21 mequiv/liter of cell water. When the same washing procedure was used with the $K^+$-containing buffer and the cells were then incubated 30 min at 37 °C, intracellular $K^+$ concentrations ranging from 25 to 80 mequiv/liter were found. When, however, the cells were washed rapidly four times with the $K^+$-containing buffer and then incubated 30 min at 37 °C, intracellular $K^+$ concentrations ranged from 143 to 188 mequiv/liter.

As shown in Fig. 1A, valinomycin almost completely blocks the reaccumulation of $K^+$ that occurs in depleted cells after addition of $K^+$ to the medium. This inhibition is partially relieved by the presence of glucose. Under these conditions, in the presence of valinomycin, glucose is nearly

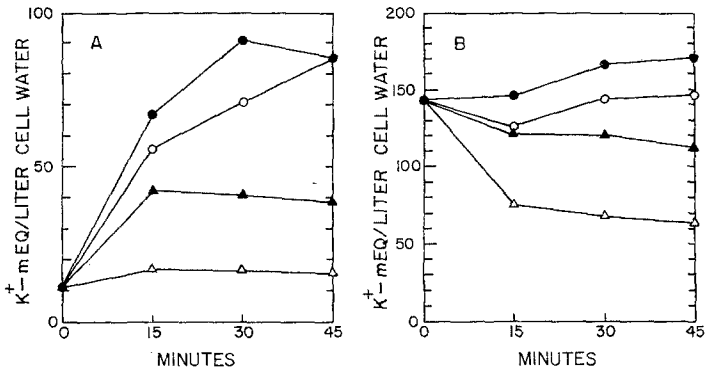


Fig. 1A and B. Effects of valinomycin and glucose, separately and together, on the $K^+$ content of Ehrlich ascites tumor cells depleted of $K^+$ and not depleted of $K^+$. In one experiment (A), the cells were depleted of $K^+$ by washing six times in a $K^+$-free buffer, and the cells were resuspended in that buffer. The addition of valinomycin to a final concentration of 10 µM and/or glucose to a final concentration of 11 mM was made immediately after sampling at zero time. KCl to a final concentration of 5 mM was added 5 min later. In another experiment (B), the cells were washed rapidly four times in a $K^+$-containing buffer and resuspended in that buffer. They were then incubated 30 min at 37 °C before the experiment was begun. Additions of valinomycin and/or glucose to the same concentrations as in A were made immediately after sampling at zero time. The suspensions in both A and B were about 20% cells. Symbols in both charts: ○ neither valinomycin nor glucose added; ● only glucose added; △ only valinomycin added; ▲ valinomycin plus glucose added

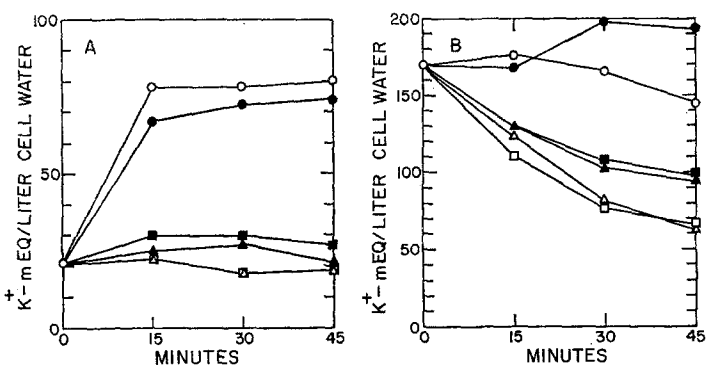


Fig. 2A and B. Effects of ouabain, with and without glucose and valinomycin, on the $K^+$ content of Ehrlich ascites tumor cells depleted of $K^+$ and not depleted of $K^+$. In one experiment (A), the cells were depleted of $K^+$ by washing six times in a $K^+$-free buffer, and the cells were resuspended in that buffer. KCl to a final concentration of 5 mM was added at zero time. The suspensions were about 20% cells. In another experiment (B), the cells were washed rapidly four times in the $K^+$-containing buffer. They were resuspended in that buffer and incubated 30 min at 37 °C before the experiment was begun. The suspensions were about 12% cells. In both A and B, glucose and the drugs were added at zero time. The additions were to the following final concentrations: glucose 11 mM, ouabain 1 mM, valinomycin 10 µM. Symbols in both charts: ○ no addition of glucose or a drug; ● only glucose added; △ only ouabain added; ▲ ouabain plus glucose added; □ ouabain plus valinomycin added; ■ ouabain plus valinomycin plus glucose added

all consumed within 15 min. No further accumulation of $K^+$ occurs after that time in the cells treated with both valinomycin and glucose. In this experiment, glucose in the absence of valinomycin appeared to accelerate the accumulation of $K^+$. However, in other experiments, such as that of Fig. 2A, this effect was not seen.

In the experiment of Fig. 1B, valinomycin caused a large loss of $K^+$ from cells originally having high intracellular $K^+$. This loss was largely prevented by the presence of glucose.

Fig. 2A shows that ouabain prevents the entry of $K^+$ into depleted cells. Glucose, valinomycin, or both do not alter this ouabain effect to any great extent. Fig. 2B shows the effect of ouabain in causing a large loss of $K^+$ from cells originally high in intracellular $K^+$. Valinomycin in combination with ouabain did not cause any additional loss. Glucose in the presence of either ouabain alone or ouabain plus valinomycin diminished the loss of $K^+$.

*Effects of $K^+$ Depletion and Repletion on Glycolysis and Intracellular pH*

Nine experiments were performed in which harvested cells were pooled and divided into two portions. The cells were washed as described above, one portion with the $K^+$-containing buffer and one with the $K^+$-free buffer. The cells washed with the $K^+$-free buffer were divided again into two portions. After all three preparations had been incubated for 5 min at 37 °C in the buffer with which they had been washed, glucose to a concentration of 11 mM was added to each. To one of the K-depleted preparations, KCl to a concentration of 5 mM was added at the same time as the glucose. Glucose and lactate were measured after 15-min incubation. There were no significant differences in utilization of glucose or production of lactate between the cells that had been washed with $K^+$-containing buffer and those to which $K^+$ was added after they had been washed in $K^+$-free buffer. However, comparison of the two preparations originally depleted of $K^+$ shows that repletion with $K^+$ increases glucose utilization and lactate production. Mean values with standard errors of the mean are expressed as μmoles/ml packed cell volume. Increased glucose utilization resulting from $K^+$ repletion was $9.3 \pm 1.1$. Increased production of lactate was $18.8 \pm 1.1$. The mean ratios of lactate production to glucose utilization were $0.99 \pm 0.06$ without $K^+$ repletion and $1.33 \pm 0.08$ with $K^+$ repletion.

Fig. 3 shows the results of an experiment in which $K^+$ was restored to the medium 2 min after addition of glucose. The lower values of $pH_e$ and $pH_i$ resulting from the addition of $K^+$ are consonant with the greater

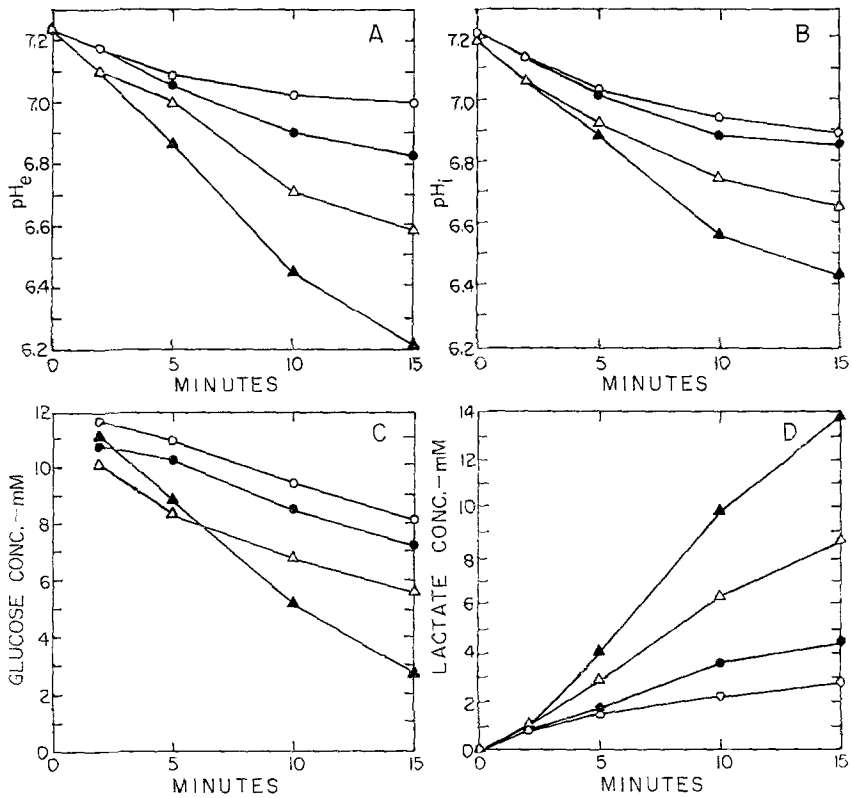


Fig. 3A–D. Effects of addition of $K^+$ alone, valinomycin alone, and $K^+$ together with valinomycin to $K^+$-depleted Ehrlich ascites tumor cells. The points designated by circles are from an experiment on one preparation of cells and those by triangles from an experiment on another batch of cells prepared in an identical manner on the following day. In each experiment the suspension of cells was divided into two parts, which were subjected to different treatments. At the beginning of the experiment, the cells were suspended in the $K^+$-free buffer. The suspensions were about 15% cells. Glucose to a final concentration of 11 mM was added to all suspensions immediately after sampling at zero time. To one suspension, no further addition was made. To the others, $K^+$, valinomycin, or both were added just after the 2-min sample was taken. The additions were made to give final concentrations of 5 mM $K^+$ and 10 μM valinomycin. Symbols: ○ no addition after glucose; ● $K^+$ alone added; △ valinomycin alone added; ▲ $K^+$ together with valinomycin added. Charts: (A) extracellular pH; (B) intracellular pH; (C) glucose concentration in the extracellular phase; (D) lactate concentration in the extracellular phase

lactate production. In each of four experiments in which $pH_i$ was measured in $K^+$-depleted glycolyzing cells, $pH_i$ fell below $pH_e$. This relationship has not been seen in glycolysis in the presence of $K^+$ in cells not treated with drugs. When the cells are repleted with $K^+$, $pH_e$ falls to a level equal to or below $pH_i$.

## *Effects of Valinomycin on Glycolysis and Intracellular pH in Cells Not Depleted of $K^+$*

Fig. 4 shows the results of an experiment in which valinomycin was added to glycolyzing cells that had been washed and resuspended in a Krebs-Ringer phosphate buffer containing 6 mM $K^+$. Valinomycin caused increases in the rates of glucose utilization and lactate production. In the presence of valinomycin, there was a greater fall of $pH_e$, corresponding to the higher concentration of lactate, and a greater fall of $pH_i$.

Ten experiments were carried out with cells washed with $K^+$-containing buffer, each preparation being divided into two portions. To both preparations, glucose was added to a concentration of 11 mM. To one, valinomycin to a concentration of $10^{-5}$ M was added at the same time as glucose. Utilization of glucose and production of lactate were measured after 15-min incubation. Mean increase in glucose utilization caused by valinomycin was $21.8 \pm 0.9$ μmoles/ml of packed cells. Mean increase in lactate production was $49.8 \pm 1.4$. The mean ratios of lactate production to glucose utilization were $1.59 \pm 0.15$ without valinomycin and $1.83 \pm 0.4$ with valinomycin.

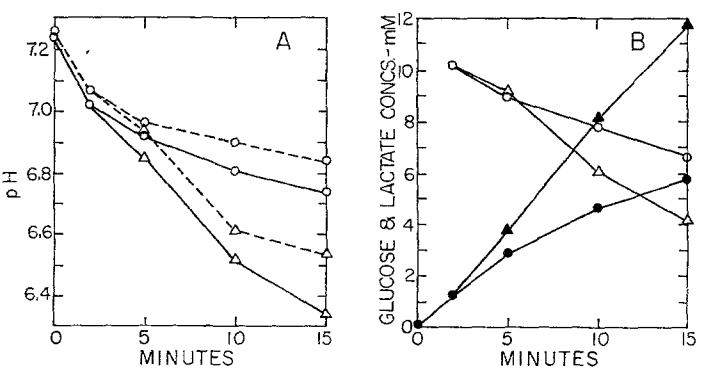


Fig. 4 A and B. Effects of valinomycin in Ehrlich ascites tumor cells washed and suspended in a Krebs-Ringer phosphate buffer of initial pH 7.35 and containing 6 mM $K^+$. The washing procedure was identical to that used with the $K^+$-free buffer in the experiment in Fig. 3. The suspension was about 15% cells. It was divided into two parts. Glucose to a final concentration of 11 mM was added to both immediately after sampling at zero time. To one, no further addition was made. To the other, valinomycin to a concentration of 10 μM was added just after the 2-min sample was taken. Symbols in A: o———o $pH_e$ in the absence of valinomycin; o-------o $pH_i$ in the absence of valinomycin; Δ———Δ $pH_e$ in the presence of valinomycin; Δ-------Δ $pH_i$ in the presence of valinomycin. Symbols in B: o———o glucose in the absence of valinomycin; Δ———Δ glucose in the presence of valinomycin; •———• lactate in the absence of valinomycin; ▲———▲ lactate in the presence of valinomycin. Glucose and lactate concentrations are measured in the extracellular phase

### *Effects of Valinomycin on Glycolysis and Intracellular pH in Cells Depleted of $K^+$*

Nine experiments were performed in which a suspension of $K^+$-depleted tumor cells was divided into two portions. Glucose alone was added to one, and glucose plus valinomycin to the other. After 15 min incubation, glucose utilization and lactate production were determined. Increased glucose utilization in the presence of valinomycin was $13.6 \pm 2.3$ μmoles/ml of packed cells. Increased lactate production in the presence of valinomycin was $29.7 \pm 3.5$. The mean ratios of lactate production to glucose utilization were $0.89 \pm 0.09$ without valinomycin and $1.41 \pm 0.10$ with valinomycin.

The results of an experiment comparing the effects of valinomycin in cells depleted of $K^+$ and repleted with $K^+$ are shown in Fig. 3. Restoration of $K^+$ enhanced considerably the effects of valinomycin on glucose utilization and lactate production.

### *Effects of Ouabain Alone and in Combination with Valinomycin on Glycolysis and Intracellular pH in $K^+$-Depleted Cells and Non-Depleted Cells*

When ouabain in a concentration of $10^{-3}$ M was added to a suspension of $K^+$-depleted cells, it almost completely blocked the augmentation of lactate production brought about by addition of $K^+$ to the medium. A concentration of $10^{-4}$ M had less effect and a concentration of $10^{-5}$ M still less. In a suspension of depleted cells in the $K^+$-free buffer to which 11 mM glucose was added, $pH_e$ fell in 30 min from 7.27 to 6.96. When $K^+$ was added with the glucose to another portion of the suspension, $pH_e$ fell from 7.28 to 6.59. When the cells were preincubated 5 min with $10^{-3}$ M ouabain, $pH_e$ fell from 7.29 to 7.05 without added $K^+$ and from 7.29 to 6.96 with added $K^+$. When $10^{-5}$ M ouabain was used, $pH_e$ fell from 7.29 to 7.05 without added $K^+$ and from 7.29 to 6.71 with added $K^+$.

In cells not depleted of $K^+$, $10^{-3}$ M ouabain largely inhibited the production of lactate from glucose. The inhibitory effect of ouabain was evident almost immediately after addition of the drug.

As shown in Fig. 5, ouabain does not obliterate the difference between $K^+$-depleted cells and non-depleted cells with respect to glucose utilization, lactate production, and changes in $pH_e$ and $pH_i$ during glycolysis. Neither does ouabain completely block the effect of valinomycin in augmenting the rate of glycolysis in depleted or non-depleted cells. Non-depleted cells treated with ouabain alone behaved very similarly to depleted cells treated with ouabain plus valinomycin.

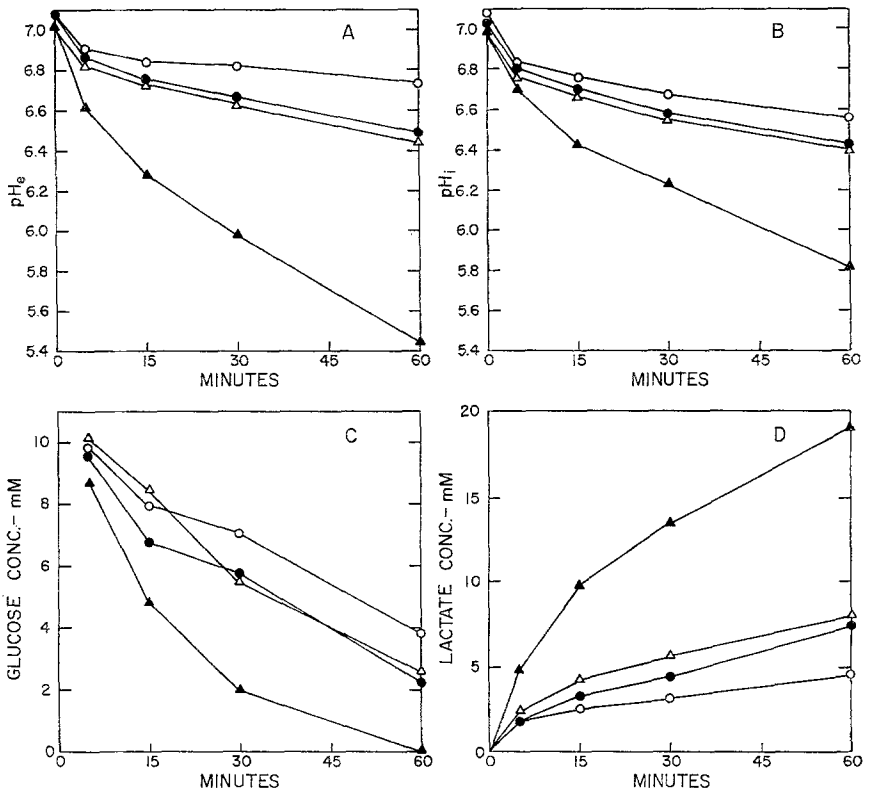


Fig. 5A–D. Effects of ouabain alone and in combination with valinomycin in Ehrlich ascites tumor cells depleted of $K^+$ and not depleted of $K^+$. Depletion was effected by washing six times in a $K^+$-free buffer, and the cells were resuspended in that buffer. The non-depleted cells were washed similarly in the $K^+$-containing buffer. They were resuspended in that buffer and incubated 30 min at 37 °C before the beginning of the experiment. The suspensions were about 25% cells. The experiments with depleted cells were performed two days before those with non-depleted cells. Ouabain to a final concentration of 1 mM was added to all suspensions 30 min before the zero time of the charts. Glucose to a final concentration of 11 mM was added to all suspensions at zero time. In the suspensions treated with valinomycin, that drug was added to a final concentration of 10 μM at zero time. Symbols: ○ ouabain alone in $K^+$-depleted cells; ● ouabain plus valinomycin in $K^+$-depleted cells; △ ouabain alone in non-depleted cells; ▲ ouabain plus valinomycin in non-depleted cells. Charts: (A) extracellular pH; (B) intracellular pH; (C) glucose concentration in the extracellular phase; (D) lactate concentration in the extracellular phase

## *Effects of $K^+$-Depletion, Valinomycin, and Ouabain on Accumulation of Glycolytic Intermediates*

In each of eight experiments in which $K^+$-depleted cells were compared with repleted cells or cells never depleted of $K^+$, the sum of the glycolytic

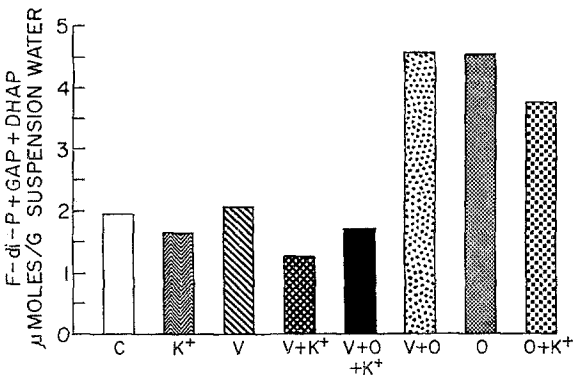


Fig. 6. Effects of valinomycin and ouabain, separately and together, on the accumulation of phosphorylated glycolytic intermediates in Ehrlich ascites tumor cells depleted of $K^+$ and not depleted of $K^+$. Depletion of $K^+$ was effected by washing six times in a $K^+$-free buffer, and the cells were resuspended in that buffer. The non-depleted cells were washed similarly in the $K^+$-containing buffer. The cells were resuspended in that buffer and incubated at 37 °C for 30 min before the experiment was begun. The suspensions were about 30% cells. In the suspensions treated with ouabain, that drug was added 30 min before zero time to a final concentration of 1 mM. Glucose was added to all suspensions at zero time to a final concentration of 11 mM. In the suspensions treated with valinomycin, that drug was added at zero time to a final concentration of 10 μM. The values shown are the sums of the concentrations of fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate. Much the greater part of this sum represents dihydroxyacetone phosphate at 30 min. Notations under the bars: *C* control; *V* valinomycin; *O* ouabain. Bars with a notation of $K^+$ represent non-depleted cells. Others represent depleted cells

intermediates, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate, accumulated to higher levels after addition of glucose in the depleted cells than in those not deficient in $K^+$. Of these three intermediates, dihydroxyacetone phosphate was always present in much higher concentrations than the others.

Fig. 6 shows the concentrations of glycolytic intermediates 30 min after the addition of glucose to $K^+$-depleted and non-depleted cells with and without treatment with valinomycin, ouabain, or both. The most marked increase in accumulation of intermediates was seen with cells treated with ouabain. Addition of valinomycin to the ouabain in depleted cells did not reduce this accumulation. The effect of ouabain was not much less in non-depleted cells than in depleted. However, addition of valinomycin as well as ouabain in non-depleted cells resulted in low levels of intermediates. Another experiment performed in the same manner as that of Fig. 6 showed the same pattern.

## Discussion

The results of our experiments showing inhibition by valinomycin of uptake of $K^+$ by $K^+$-depleted Ehrlich tumor cells are in agreement with the reports of Levinson (1967) and Levinson and Hempling (1967). The fact that glucose was consumed early in the experiment of Fig. 1 probably accounts for the reversal of the valinomycin inhibition by glucose appearing to be temporary rather than complete. The depletion of cellular ATP brought about by the uncoupling action of valinomycin reported by Levinson (1967) can satisfactorily account for the inhibition of the transport system involved in the reaccumulation of $K^+$ into depleted cells, as has been suggested by Levinson. It can also account for the effect of valinomycin in causing loss of $K^+$ from high-$K^+$ cells shown in the experiments reported here. The action of glucose in reversing these effects of valinomycin both in depleted and non-depleted cells may be explicable in terms of the fact that ATP generated by glycolysis can be utilized for cation transport.

The effect of ouabain in blocking the uptake of $K^+$ by depleted cells, as found in our experiments, is also in agreement with the reports of Levinson (1967) and Levinson and Hempling (1967). Our experiments showing loss of $K^+$ from non-depleted cells brought about by ouabain confirm the studies of Bittner and Heinz (1963). The effects of ouabain both in depleted and non-depleted cells were not altered by addition of valinomycin. Unlike Levinson, we have found that glucose reverses to a small extent the effects of ouabain on $K^+$ movement both in depleted and non-depleted cells. The additional ATP generated by glucose metabolism may partially relieve the inhibition of the transport enzyme by furnishing an increased amount of substrate.

Our experiments showing impaired capacity of the $K^+$-depleted Ehrlich ascites tumor cell to produce lactic acid from glucose and the rapid restoration, at least in part, of that capacity by addition of $K^+$ to the external medium are in agreement with the reports of Maizels *et al.* (1958*b*) and of Gordon and de Hartog (1968).

It would appear that the glycolytic disturbances in $K^+$-depletion are the consequence of more than one factor. Gordon and de Hartog (1968) favored the view that the effect is at the plasma membrane and that it involves the active transport of $Na^+$ and $K^+$ across that membrane. Some of our experimental results support this idea. An inhibitory effect of ouabain on lactate production in high-$K^+$ cells was seen almost immediately after addition of the drug before there had been time for much loss of intra-

cellular $K^+$. A possible mechanism through which ouabain might inhibit glycolysis by an action at the plasma membrane involves the accumulation of ATP, which is not being utilized by the inhibited ATPase. This increased concentration of ATP in turn would inhibit glycolysis.

On the other hand, some of our experiments furnish evidence that the intracellular $K^+$ concentration of itself is also a factor in determining the rate of glycolysis. As shown in Fig. 5, when transport is inhibited by ouabain, glycolysis proceeds at a faster rate in cells with high intracellular $K^+$ than in those with low intracellular $K^+$. It is not surprising that low intracellular $K^+$ should inhibit glycolysis and cause abnormal accumulation of intermediates in that some of the glycolytic enzymes, such as pyruvate kinase, are activated by $K^+$. The molar concentration of $K^+$ required for maximum activity is, in most cases, high (Suelter, 1970).

The effect of ouabain in blocking the stimulation of glycolysis on addition of $K^+$ to depleted cells could be explained equally well in terms of either of the two mechanisms proposed above.

The stimulation of glycolysis by valinomycin in non-depleted Ehrlich tumor cells is in agreement with the reports of Wenner *et al.* (1967) and of Gordon and de Hartog (1968). Our finding that valinomycin stimulated glycolysis even in $K^+$-depleted cells is in contrast to the report of Gordon and de Hartog. However, the concentration of valinomycin in our experiments was much greater than that used by those workers. We did find that valinomycin had a much greater effect in repleted or non-depleted cells than in depleted cells, as shown in Figs. 3 and 4.

In all of the experiments reported here, the fall of $pH_e$ agrees with that calculated from the lactate concentration. This has been found in earlier experiments with glycolyzing cells (Poole, 1967). With none of the treatments used in the present experiments is there evidence of any significant source of protons other than lactic acid contributed to the external medium after the initial time of suspension.

As shown in Fig. 5, the stimulation of glycolysis caused by valinomycin is not prevented by ouabain. Neither the additional ATP generated by the increased rate of glycolysis caused by valinomycin nor the ATP spared by inhibition by ouabain of the membrane cation pump is sufficient to inhibit glycolysis in the presence of valinomycin. One possibility is that ATP is being consumed in the energy-requiring transport of $K^+$ into mitochondria mediated by valinomycin.

As shown in Fig. 6, in every matched pair, $K^+$-depletion was associated with an increased level of glycolytic intermediates, principally dihydroxy-

acetone phosphate. Much the highest levels are found in the presence of ouabain except where valinomycin is also present in the high-$K^+$ cells. In the latter condition, inhibition of glycolysis is also largely overcome.

The unusual relationship in which $pH_i$ values are lower than those of $pH_e$ in glycolyzing $K^+$-depleted cells or ouabain-treated depleted or non-depleted cells may be explained in terms of little lactate production to lower $pH_e$ together with intracellular accumulation of phosphorylated intermediates. This is a situation resembling that seen in Ehrlich tumor cells incubated with 2-deoxyglucose (Poole, 1967) or with glucose in the presence of iodoacetate or oxamate (Poole & Butler, 1969). The role of phosphorylation in intracellular acidification is discussed in those two reports.

The greater lowering of $pH_e$ when cells are glycolyzing in the presence of valinomycin is associated with a greater lowering of $pH_i$. With or without valinomycin, the relationship of $pH_i$ to $pH_e$ is about the same as had been observed in non-glycolyzing cells suspended in phosphate buffers (Poole *et al.*, 1964). The greater lowering of $pH_i$ in the presence of valinomycin is simply the result of the greater lowering of $pH_e$. Valinomycin resembles other uncoupling agents in its effect in stimulating glycolysis, but it differs from such agents as 2,4-dinitrophenol and bishydroxycoumarin in that it does not abolish the pH gradient across the plasma membrane (Poole, 1968). It has been the conclusion of several investigators (*e.g.* Carafoli, Rossi, & Gazzotti, 1969; Henderson, McGivan, & Chappell, 1969) that valinomycin increases the permeability of membranes to $K^+$ but not to $H^+$, whereas uncouplers such as dinitrophenol increase permeability to $H^+$. However, Poole (1968) found that the $pH_e$–$pH_i$ gradient was abolished only in glycolyzing cells, not in non-glycolyzing cells. Since the effects of the uncouplers could not be accounted for as a general increase in permeability of the plasma membrane to $H^+$, it was suggested that these agents might render the membrane permeable to the lactate ion.

In none of our experimental conditions was all of the glucose utilized accounted for as lactate. However, the increased utilization of glucose resulting from addition of $K^+$ to $K^+$-depleted cells or addition of valinomycin either to $K^+$-depleted or non-depleted cells was entirely accounted for by the increased lactate production, the ratio of extra lactate production to extra glucose utilization being approximately 2 in each case. This would seem to indicate that pathways of glucose metabolism other than lactate production are insensitive to depression by changes of ionic composition or stimulation by valinomycin.

## References

Bittner, J., Heinz, E. 1963. Die Wirkung von *g*-Strophantin auf den Glyzintransport in Ehrlich-Ascites-Tumorzellen. *Biochim. Biophys. Acta* **74**:392.

Carafoli, E., Rossi, C. S., Gazzotti, P. 1969. The effect of uncoupling agents and detergents on the movements of monovalent cations between mitochondria and medium. *Arch. Biochim. Biophys.* **131**:527.

Gordon, E. E., de Hartog, M. 1968. Valinomycin-stimulated glycolysis in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **162**:220.

– Nordenbrand, K., Ernster, L. 1967. Evidence for a new mechanism of respiratory stimulation and proton ejection in Ehrlich ascites tumour cells dependent on potassium ions. *Nature* **213**:82.

Henderson, P. J. F., McGivan, J. D., Chappell, J. B. 1969. The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. The role of induced proton permeability. *Biochem. J.* **111**:521.

Levinson, C. 1967. Effect of valinomycin on net sodium and potassium transport in Ehrlich ascites tumour cells. *Nature* **216**:74.

– Hempling, H. G. 1967. The role of ion transport in the regulation of respiration in the Ehrlich mouse ascites tumor cell. *Biochim. Biophys. Acta* **135**:306.

Maizels, M., Remington, M., Truscoe, R. 1958*a*. Data for the calculation of the rate coefficients of sodium transfer by mouse ascites tumour cells. *J. Physiol.* **140**:48.

– – – 1958*b*. The effects of certain physical factors and of the cardiac glycosides on sodium transfer by mouse ascites tumour cells. *J. Physiol.* **140**:61.

McMurray, W. C., Begg, R. W. 1959. Effect of valinomycin on oxidative phosphorylation. *Arch. Biochem. Biophys.* **84**:546.

Moore, C., Pressman, B. C. 1964. Mechanism of action of valinomycin on mitochondria. *Biochem. Biophys. Res. Commun.* **15**:562.

Poole, D. T. 1967. Intracellular pH of the Ehrlich ascites tumor cell as it is affected by sugars and sugar derivatives. *J. Biol. Chem.* **242**:3731.

– 1968. The effect of uncoupling agents on the pH gradient across the plasma membrane of the Ehrlich ascites tumor cell. *Biochem. Biophys. Res. Commun.* **32**:403.

– Butler, T. C. 1969. Effects of inhibitors of glycolysis on intracellular pH and on accumulation of glycolytic intermediates in the Ehrlich ascites tumor cell. *J. Nat. Cancer Inst.* **42**:1027.

– – Waddell, W. J. 1964. Intracellular pH of the Ehrlich ascites tumor cell. *J. Nat. Cancer Inst.* **32**:939.

Post, R. L., Merritt, C. R., Kinsolving, C. R., Albright, C. D. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. Biol. Chem.* **235**:1796.

Schatzmann, H.-J. 1953. Herzglykoside als Hemmstoffe für den aktiven Kalium- und Natriumtransport durch die Erythrocytenmembran. *Helv. Physiol. Acta* **11**:346.

Suelter, C. H. 1970. Enzymes activated by monovalent cations. *Science* **168**:789.

Waddell, W. J., Butler, T. C. 1959. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *J. Clin. Invest.* **38**:720.

Wenner, C. E., Harris, E. J., Pressman, B. C. 1966. Relationship of the light scattering properties of mitochondria to the metabolic state in intact ascites cells. *Biophys. J.* **6**:40 abs.

– – – 1967. Relationship of the light scattering properties of mitochondria to the metabolic state in intact ascites cells. *J. Biol. Chem.* **242**:3454.