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VALINOMYCIN-STIMULATED GLYCOLYSIS IN EHRLICH ASCITES TUMOR CELLS*

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SUMMARY

K^+ transport across cell membrane and mitochondrial membrane exerted a profound influence on glycolysis in a model system consisting of K^+-depleted Ehrlich ascites tumor cells.

1. Initiation of K^+ transport across the cell membrane by addition of K^+ to depleted cells resulted in a 2–3-fold increase in the rate of glycolysis. Substitution of K^+ with Rb^+, but not with Cs^+, resulted in an identical response. Inhibition of cell membrane ATPase with ouabain almost completely abolished this stimulation to glycolysis.

2. Valinomycin-induced mitochondrial K^+ transport in intact cells enhanced the rate of glycolysis 6–8 times over control values and abolished the Crabtree effect. The response to valinomycin was seen when either Rb^+ or Cs^+ was substituted for K^+. The effects of mitochondrial K^+ transport were apparent when the cell membrane mechanism was inhibited with ouabain.

3. The transport of K^+ across cell membrane and across mitochondrial membrane were equally effective in stimulating glycolysis in the tumor cells.

4. Valinomycin was without effect on erythrocyte glycolysis.

INTRODUCTION

The energy-dependent transport of K^+ across the cell membrane functions as a mechanism for regulating the rate of glycolysis. Activation of K^+ transport across the cell membrane of intact erythrocytes stimulates the oxidation of intermediates through the Embden–Meyerhof pathway[1,2]. Interference with K^+ transport by ouabain, an inhibitor of the Na^+–K^+-dependent ATPase, diminishes the rate of erythrocyte glycolysis[1,3,4]. This interrelationship between glycolysis and active K^+ transport is thought to be mediated *via* the synthesis and degradation of ATP.

Recently, another energy-dependent K^+ transport system has been described with isolated mitochondria[5]. In contrast to the cell membrane mechanism, transport of K^+ into mitochondria requires the dodecadepsipeptide antibiotic, valinomycin, is independent of the presence of ATP and Na^+, and is insensitive to ouabain. Both cell membrane and mitochondrial K^+ transport have been shown to operate in intact

* A portion of this work has appeared in abstract form[31].

Ehrlich ascites tumor cells[6]. The functional significance of this mitochondrial cation transport system has remained obscure. In the present experiments, data are presented which demonstrate a role for mitochondrial cation transport in the intact cell: a marked stimulation of glycolysis ensues when mitochondrial K^+ transport is initiated with valinomycin. Moreover, this effect of mitochondrial K^+ transport on glycolysis can be seen in the presence or in the absence of the energy-dependent transport of K^+ across the cell membrane.

METHODS

Ehrlich ascites tumor cells of the hyperdiploid strain (initially provided by Dr. GUSTAV DALLNER, Stockholm) were harvested from the peritoneal cavity of white mice 6–10 days after inoculation. K^+ depletion was accomplished by collecting and repeatedly washing the tumor cells in a K^+-free medium at 4° as previously described[6]. The concentration of K^+ in such depleted cells is approx. 2–4 mequiv/l as has been previously reported[7]. Non-depleted cells were prepared in a similar manner except that 5 mM K^+ was present in the bathing fluid. Human erythrocytes were prepared from heparinized blood drawn in the post-absorptive state as described by CHAPMAN *et al.*[8].

All incubations were carried out at 37° in an air atmosphere. H^+ production by tumor cells was monitored at 30-sec intervals by determination of the amount of 0.005 M NaOH (in 0.9 % NaCl) required to titrate the incubate to pH 7.4. The Radiometer pH meter 26 and Titrator 11 were used for this purpose. The concentrations of ions in the medium used for the titration experiments were: Na^+ 159, Mg^{2+} 1.5, Cl^- 155, SO_4^{2-} 1.5, and PO_4^{3-} 2 mmoles/l; approx. 40 mg of cell protein were present in an initial volume of 6 ml. In those experiments where cell respiration was measured, approx. 20 mg of cell protein were present in a final volume of 3 ml. The O_2 content of the incubation medium was determined with the Clark Electrode and continuously charted on a Varian G-11 recorder. The incubation medium was identical to that used for the titration experiments except that PO_4^{3-} was present at a concentration of 12 mmoles/l and Na^+ at a concentration of 179 mmoles/l. The erythrocyte suspensions were incubated for 1 h in a Dubnoff metabolic shaker. The pH of the cell suspension was adjusted with 0.3 M glycine buffer so that the pH after incubation was between 7.6 and 7.8. All incubations were terminated at the appropriate times by addition of $HClO_4$. The protein free filtrates were analyzed for glucose with Glucostat reagent (glucose oxidase) and for lactate with lactate dehydrogenase[9]. The protein content of the tumor cell suspension was determined by a biuret technique.

RESULTS

Influence of K^+ and valinomycin on glycolysis

When Ehrlich cells are incubated with glucose, the high rate of glycolysis and relative inability to oxidize pyruvate *via* the tricarboxylic acid cycle is manifested by an almost stoichiometric conversion of glucose to lactic acid[10]. In the absence of exogenous substrate a slow release of H^+ is observed with K^+-depleted Ehrlich ascites tumor cells (Fig. 1, Curve A) as well as with non-depleted cells[6]. The small amount of lactic acid produced under these conditions can not account for the observed release

of H^+. Upon addition of glucose to the incubation medium, there is a prompt and pronounced increase in hydrogen ion release which lasts for about 1 min (Fig. 1, Curve B). Then a relative inhibition of hydrogen ion release is observed and the rate of release thereafter remains fairly constant over the time period studied as has been previously reported[11,12].

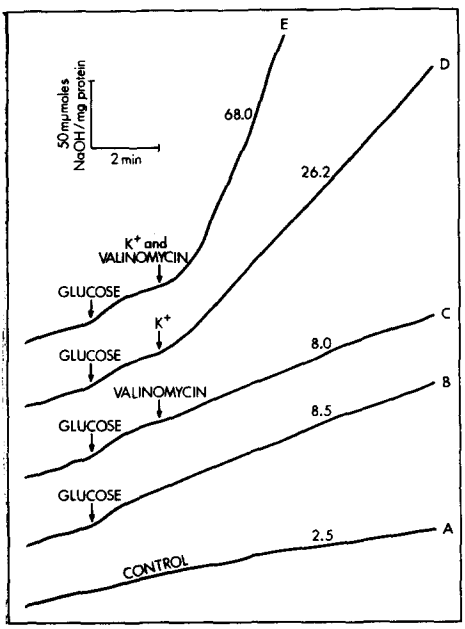


Fig. 1. The effect of K^+ and valinomycin on H^+ release by K^+-depleted cells. The initial volume of the incubation medium was 6.0 ml. In this and all subsequent experiments the initial concentration of glucose was 5 mM. K^+ (30 μmoles) and valinomycin (10^{-3} μmole) were added as indicated. The values adjacent to the curves are rates in mμmoles H^+ produced per mg protein per min.

Addition of valinomycin to the K^+-depleted cells is without effect on the rate of hydrogen ion release in a glucose-containing medium (Fig. 1, Curve C). This finding is compatible with the known K^+ requirement for valinomycin action on isolated mitochondria[5] and for stimulation of hydrogen ion release in intact K^+-depleted Ehrlich ascites tumor cells metabolizing endogenous substrate[6]. Activation of the cell membrane K^+ transport by addition of potassium to the depleted cells metabolizing glucose results in an immediate and marked stimulation of the rate of hydrogen ion production (Fig. 1, Curve D). H^+ production remains constant at about three times the rate observed in the absence of K^+.

Stimulation of mitochondrial K^+ transport by addition of K^+ *plus* valinomycin to K^+-depleted cells increases hydrogen ion release by approximately 8-fold (Fig. 1, Curve E). This stimulatory effect is observed within 30 sec after addition of K^+ and valinomycin and persists for the duration of the experimental observation period. An increase in H^+ ejection upon addition of valinomycin to ascites tumor cells metabolizing glucose has been observed by WENNER, HARRIS AND PRESSMAN[13]. However, in their experiments no attempt was made to demonstrate the K^+ dependence of the valinomycin effect.

TABLE I

RELATIONSHIP BETWEEN PROTON RELEASE AND LACTATE PRODUCTION BY TUMOR CELLS IN PRESENCE OF GLUCOSE

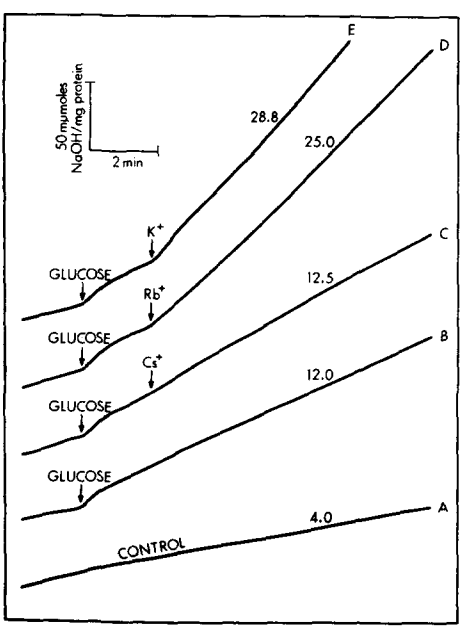
The values are corrected for the rate of proton release in the absence of glucose, and are the means ± S.E. of 7 experiments. The concentration of additions were as indicated in the legend to Fig. 1.

Additions	*Net proton release* (*μmoles/ml cells*)	*Lactate accumulation* (*μmoles/ml cells*)
Control	3.71 ± 0.42	3.01 ± 0.30
Valinomycin	3.51 ± 0.28	2.86 ± 0.46
K^+	7.82 ± 0.44	6.41 ± 0.41
Valinomycin + K^+	17.04 ± 1.18	14.52 ± 0.75

There is a close correlation between total hydrogen ion release and lactic acid accumulation during the incubation (Table I). Therefore, under the conditions of these experiments and also when rubidium and cesium ions were substituted for K^+, hydrogen ion release is equated with lactic acid production.

Influence of Rb^+ and Cs^+ on glycolysis

The ATPase system associated with the cell membrane and endoplasmic reticulum is thought to be involved in the active transport of monovalent cations and requires the presence of Mg^+, Na^+ and K^+. Enzyme activity is retained when K^+



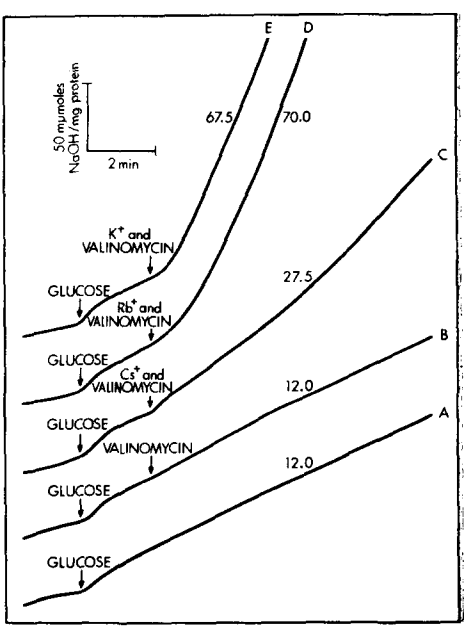


Fig. 2. Effect of Rb^+ and Cs^+ on the rate of lactic acid formation by K^+-depleted cells. Conditions as indicated in legend to Fig. 1.

Fig. 3. Effect of valinomycin on the rate of lactic acid formation after addition of Rb^+ or Cs^+ to K^+-depleted cells.

is replaced with Rb^+. Replacement of K^+ with Cs^+ markedly reduces activity in some systems[14], but not in others[15]. In the intact K^+-depleted Ehrlich ascites tumor cell, an increased respiratory response and proton release is observed upon addition of K^+ or Rb^+, but not when Cs^+ is added[6]. A study of the cation specificity on the glycolytic response of K^+-depleted Ehrlich ascites tumor cells reveals an identical pattern (Fig. 2). Addition of K^+ (Curve E) or Rb^+ (Curve D) to the cell suspension results in an immediate 2–3-fold increase in lactate production; Cs^+ (Curve C) has no effect on lactate production.

In contrast to the specific cation requirements for the coupled Na^+–K^+ cell membrane transport mechanism, either Rb^+ or Cs^+ is able to substitute for K^+ in the valinomycin-stimulated cation–proton exchange in isolated mitochondria[5]. Both Rb^+ and Cs^+ are also capable of replacing K^+ in the demonstration of the valinomycin-stimulated respiration and proton release in intact Ehrlich ascites tumor cells[6]. Similarly, stimulation of glycolysis by valinomycin is observed when either K^+, Rb^+ or Cs^+ are added to the medium (Fig. 3). The contribution of Cs^+ *plus* valinomycin results in an immediate 2–3-fold increase in glycolysis (Curve C). Valinomycin in the presence of either Rb^+ (Curve D) or K^+ (Curve E) results in a gradual increase in glycolysis over the first minute or two, and then a more pronounced increase of 5–6-fold over the control value which remains constant over the period of observation.

Influence of ouabain on glycolysis

The increase in lactate production following addition of K^+ to K^+-depleted cells (Fig. 1, Curve D) could have been due to the reestablishment of an appreciable intracellular K^+ concentration rather than to cell membrane transport of K^+. To answer this question, K^+-containing Ehrlich ascites tumor cells were prepared and incubated in a K^+-containing medium. Since ouabain blocks the Na^+–K^+ coupled cell membrane ATPase transport system[14], inhibition of cell membrane K^+ transport with ouabain would be expected to have little effect on the rate of glycolysis if restoration of the intracellular K^+ were the important factor. The data presented in Fig. 4 show that inhibition of plasma membrane transport of K^+ with non-depleted cells reduces the utilization of glucose ($P < 0.01$) and the production of lactate ($P < 0.001$). The actual increment in glycolysis resulting from cell membrane transport of K^+, *i.e.*, the control rate *minus* the rate in the presence of ouabain, is 2.6 mμmoles glucose utilized per mg protein per min and 7.8 mμmoles lactate produced per mg protein per min. This observation supports the concept that the dominant factor governing the increase in glycolysis following K^+ addition to depleted cells is the transport of K^+ across the cell membrane rather than the increase in intracellular K^+ concentration.

The stimulation of glycolysis which occurs in the presence of valinomycin and K^+ represents the summation of the effects of cell membrane K^+ transport and mitochondrial K^+ transport. Although valinomycin selectively increases the permeability of the red blood cell membrane to K^+, the Na^+ pump and the $Na^+ + K^+$ ATPase activity are relatively uneffected[16]. Therefore, dissociation of the cell membrane and mitochondrial contributions is possible by inhibiting only the cell membrane transport system with ouabain in the presence of valinomycin (Fig. 4). With K^+-containing cells, in the absence of ouabain, the net increase in the glycolytic rate due to valinomycin is 16.9 mμmoles glucose utilized per mg protein per min and 23.4 mμmoles

lactate produced per mg protein per min. These derived values for the effects of mitochondrial K^+ transport in non-depleted cells are somewhat higher than the net effect of valinomycin addition when the cell membrane mechanism is inhibited with ouabain, *i.e.*, 11.3 mμmoles of glucose utilized per mg protein per min and 17.8 mμmoles of lactate produced per mg protein per min.

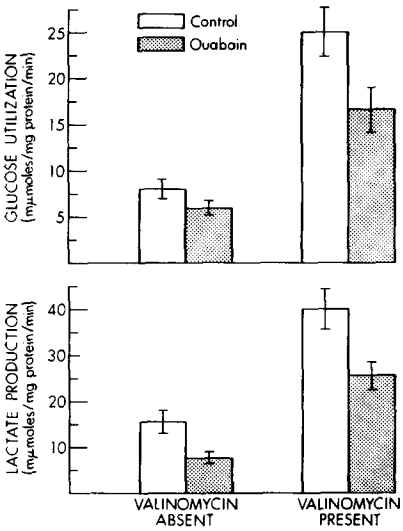


Fig. 4. Influence of ouabain on valinomycin-stimulated glycolysis in K^+-containing cells. The S.E. of the mean is indicated by a vertical bar. Ouabain was present in the 3 ml incubation at a final concentration of 10^{-3} M. The final concentration of valinomycin was $1.6 \cdot 10^{-7}$ M. The incubation was terminated after 10 min by addition of perchloric acid.

In confirmation of the data obtained by titration experiments (Fig. 1), a 2–3-fold increase in glucose utilization and lactic acid production is observed following addition of K^+ to K^+-depleted cells (Table II). Ouabain is without effect on K^+-depleted cells, but almost completely inhibits the increment in the rate of glycolysis seen upon K^+ repletion. Valinomycin addition has little effect on K^+-depleted cells, but stimulates glycolysis 5–6-fold when cells are repleted with K^+ in agreement with the data presented in Figs. 1 and 3. The stimulatory effect of valinomycin on K^+-

TABLE II

GLYCOLYSIS IN K^+-DEPLETED AND K^+-REPLETED CELLS

The values are in mμmoles/mg protein per min $\pm$ S.E. of 8 experiments.

Additions	*K^+-depleted*		*K^+-repleted*	
	Glucose utilized	*Lactate produced*	*Glucose utilized*	*Lactate produced*
Control	3.4 $\pm$ 0.6	5.6 $\pm$ 0.5	10.4 $\pm$ 1.1	15.9 $\pm$ 0.9
Ouabain	2.9 $\pm$ 0.7	5.4 $\pm$ 0.4	4.7 $\pm$ 0.5	7.2 $\pm$ 0.5
Valinomycin	5.4 $\pm$ 0.3	6.0 $\pm$ 0.4	24.8 $\pm$ 2.1	39.8 $\pm$ 1.7
Ouabain + valinomycin	4.1 $\pm$ 0.8	5.8 $\pm$ 0.5	13.3 $\pm$ 1.5	18.7 $\pm$ 1.3

repleted cells is partially inhibited in the presence of ouabain. Roughly half of the increase in glycolysis in the presence of K^+ and valinomycin is due to cell membrane K^+ transport and the other half to the effect of valinomycin on mitochondrial metabolism. This contrasts with a far lesser influence of cell membrane K^+ transport on glycolysis in cells containing K^+ (Fig. 4).

Effect of glucose, K^+ and valinomycin on respiration of K^+-depleted cells

Concurrent with studies of glycolysis, oxidative metabolism was followed by continuously monitoring the oxygen uptake of K^+-depleted and K^+-repleted cells in the presence and absence of valinomycin and glucose (Table III). Addition of glucose to K^+-depleted cells (Expt. 1), to K^+-repleted cells (Expt. 2), and to K^+-depleted cells *plus* valinomycin (Expt. 3) results in a significant depression of respiration. This phenomenon, first described by CRABTREE[17] can, however, be blocked by

TABLE III

RESPIRATION AND CRABTREE EFFECT OF K^+-DEPLETED CELLS

O_2 uptake in mμmoles/mg protein per min. Mean ± S.E. of 5 experiments. In each experiment the agents were added sequentially.

	Qo_2
Expt. 1	
Control	3.7 ± 0.3
+ glucose	2.8 ± 0.1
Expt. 2	
Control	3.7 ± 0.2
+ K^+	4.8 ± 0.4
+ glucose	3.0 ± 0.1
Expt. 3	
Control	3.5 ± 0.2
+ valinomycin	3.9 ± 0.3
+ glucose	3.0 ± 0.1
Expt. 4	
Control	3.5 ± 0.2
+ K^+ + valinomycin	5.2 ± 0.5
+ glucose	5.0 ± 0.4

addition of the uncoupler, dinitrophenol[12]. The combination of K^+ and valinomycin (Expt. 4) similarly blocks the Crabtree effect, presumably by draining off a high energy intermediate for transport of K^+ into the mitochondria. The stimulation of respiration upon addition of K^+ to depleted cells (Expt. 2) confirms previous observations[6] and is consistent with WHITTAM's hypothesis[18] that monovalent cation transport across the cell membrane influences the rate of electron transport through the mitochondrial respiratory chain. In the absence of added K^+, valinomycin is without effect on the respiratory rate (Expt. 3) as seen with isolated mitochondria[5] and as observed earlier with intact Ehrlich ascites tumor cells[6]. When K^+ is present, however, valinomycin addition results in an enhanced respiration (Expt. 4).

Erythrocyte glycolysis

Although the response of intact ascites tumor cells to valinomycin paralleled that seen with isolated mitochondria, valinomycin has also been shown to enhance the permeability of sheep red blood cells to potassium[16]. Because of this observation, the rate of glycolysis of erythrocytes suspended in a medium containing potassium was examined in the presence and in the absence of valinomycin. Neither glucose utilization (control 10.4, valinomycin 10.4 mμmoles per g hemoglobin per h) nor lactate production (control 14.2, valinomycin 15.1 mμmoles per g hemoglobin per h) are influenced by valinomycin.

DISCUSSION

The K^+-depleted Ehrlich ascites tumor cell is a model system for studying the effects of K^+ and K^+ transport on glycolysis under particularly favorable circumstances. The characteristics of the cell membrane and the mitochondrial K^+ transport systems provide a means for identifying and functionally separating these two systems in intact, respiring cells[6].

The characteristics of the cell membrane transport of K^+ in Ehrlich ascites tumor cells are qualitatively identical to those reported for other cell types. The inward transport of K^+ is coupled with the Na^+ pump, is ATP dependent, and is inhibited by ouabain. Stimulation of K^+ transport across the cell membrane is accomplished by first depleting the cells of K^+ by repeated washings in the cold, and then incubation of cells in a K^+-containing medium at 37°. In the absence of exogenous substrate there is a marked increase in respiration[6] and accumulation of intracellular potassium[19] providing that Na^+ is present in the medium and ouabain is deleted. The rate of oxidation of glucose to lactate is accelerated about 2–3-fold upon stimulation of cell membrane K^+ transport. The enhanced rate is observed immediately upon addition of K^+ and remains at a high constant level as K^+ continues to accumulate within the cell. It is for these reasons and also because ouabain inhibits glycolysis in cells that have not been depleted of K^+, that the regulation of glycolysis is a consequence of K^+ transport rather than re-establishment of a high intracellular K^+ concentration. A similar inhibiting effect of ouabain on glycolysis has been reported for kidney tissue[20], erythrocytes[1,4], ciliary body[21], and muscle[22].

The mechanism by which active plasma membrane K^+ transport regulates the rate of glycolysis is not clear. It is generally held that there are several rate limiting steps in the oxidation of glucose to lactate. In ascites tumor cells, phosphofructokinase appears to be the principal site where control is exercised over the rate of glycolysis[23,24]. The activity of the phosphofructokinase enzyme is particularly sensitive to adenine nucleotides and inorganic phosphate; ATP inhibits and ADP and P_i stimulate the activity of this enzyme[25]. Thus, a feed-back mechanism exists whereby ATP produced through glycolysis or *via* the respiratory chain blocks glycolysis. During the course of active K^+ transport across the plasma membrane, ATP is hydrolyzed to ADP and P_i. The transport process therefore provides optimal conditions for phosphofructokinase activity which in turn theoretically stimulates glycolysis. The ATP generated as a result of glycolysis could then be utilized to support cell membrane cation transport as suggested by WHITTAM AND AGER[1]. On the other hand, PARKER AND HOFFMAN[2] have suggested that phosphoglycerate kinase is the specific site where active

cation transport influences glycolysis. The results obtained in the present study of Ehrlich ascites tumor cells are consistent with either of these hypotheses.

The stimulation of mitochondrial K^+ transport by addition of valinomycin results in a marked increase in the rate of glycolysis. Either K^+, Rb^+ or Cs^+ must be added to K^+-depleted cells in order to observe the stimulation of glycolysis by valinomycin. Therefore, the same cation specificity holds for demonstration of the valinomycin effect on respiration and protein extrusion in the intact cell[6] and for detection of energy-dependent monovalent cation uptake in isolated mitochondria[5]. In the presence of valinomycin and the appropriate cation, mitochondria of the intact cell have been shown to imbibe H_2O and swell when studied by light scattering techniques[13] and by electron microscopy*.

Apparently valinomycin also permits the diffusion of K^+ across the cell membrane in the direction of an electrochemical gradient[16]. This observation is consistent with the non-biological interaction of K^+ with valinomycin in the presence of lipid bilayers[26,27] and in the absence of lipid[28,29]. If valinomycin permitted passive diffusion of K^+ across the cell membrane of the ascites tumor cell, the characteristic effects of valinomycin on mitochondrial metabolism would be expected upon addition of K^+ to the depleted cells, even when active K^+ transport is inhibited with ouabain. In fact, this is what is observed for both valinomycin-stimulated respiration and proton extrusion in endogenously metabolizing cells[6] and for valinomycin-stimulated glycolysis after addition of glucose. The cell membrane monovalent cation pump is capable of restoring toward normal the intracellular cation concentration in the presence of glucose despite the valinomycin-induced passive cation diffusion[19] as would be anticipated from the experiments of TOSTESON and coworkers[16].

The mechanism by which valinomycin-stimulated mitochondrial K^+ transport affects glycolysis is not clear. However, operation of the mitochondrial K^+ transport system results in diminished intracellular ATP[19] and presumably elevated levels of ADP and P_i, conditions that favor glycolysis. Mitochondrial ATP synthesis is effectively blocked by K^+ *plus* valinomycin because of the shunting of a high energy intermediate(s) prior to ATP formation for support of the K^+ transport system. Since valinomycin is also known to activate mitochondrial ATPase[30] it is possible that ATP generated during glycolysis would be hydrolyzed as has been previously suggested[19].

These data clearly demonstrate that K^+ transport serves to regulate the rate of glycolysis in intact, respiring cells. The magnitude of the changes in the rate of glycolysis upon stimulation or inhibition of the cell membrane mechanism suggests that this is an important controlling device in intact cells. Further, the data point up one of the physiological consequences of mitochondrial cation transport. The importance of this latter mechanism for metabolic regulation awaits the demonstration and effectiveness of naturally occurring agents which behave like valinomycin.

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