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EFFECT OF VALINOMYCIN ON ION TRANSPORT IN BACTERIAL CELLS AND ON BACTERIAL GROWTH

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SUMMARY

The antimicrobial action of valinomycin relative to the K^+ and Na^+ contents of the medium has been investigated in several species of bacteria, particularly in *Streptococcus faecalis*, which effects energy-linked transport exclusively via degradation of glycolytic ATP, *Micrococcus lysodeikticus*, effecting active ion transport by respiration and *Staphylococcus aureus*, the energy-dependent ion transport of which is due to both glycolytic ATP degradation and respiration. It was demonstrated that valinomycin does not act on K^+ transport in the glycolysing cells in the same manner as it does on respiring cells under similar conditions. Addition of valinomycin to respiring cells leads to an increase in K^+ influx against the concentrational gradient in both growing and resting cells. In contrast to this, antibiotic-treated glycolysing cells experience passive K^+ outflow down the concentrational gradient. It was thus concluded that the electrical potential cannot be the driving force for the energy-linked K^+ transport in glycolysing cells.

INTRODUCTION

Physiologically active compounds increasing the ion permeability of membranes are at present attracting considerable attention. Foremost among them is valinomycin, a cyclic depsipeptide that forms positively charged lipophilic complexes with alkali metal ions and selectively affects the membrane permeability. Valinomycin has become a useful tool in the study of ion transport in biological systems.

The effects of this antibiotic on ion transport in intact cells and on the growth of the bacteria has been extensively studied by Harold et al. [1–3] on *Streptococcus faecalis* 9790 which, possessing a reduced oxidative phosphorylation system, has all its energy-dependent processes relying on glycolytic ATP. Harold et al. [1] showed that valinomycin inhibited *S. faecalis* growth only at low K^+ contents in the medium and at a pH 6.0, growth inhibition by the antibiotic being nullified at higher extracellular K^+ concentrations or on increasing the pH to 7.5. Growth inhibition was accompanied by passive K^+ efflux down the concentration gradient in exchange for protons of the medium, the exchange being intensified by the addition of proton conductors.

The effect of valinomycin on ion transport in the cells of the obligate aerobes *Azotobacter vinelandii O* and *Mycobacterium phlei* has been investigated by Pressman and Hempfling [4], who showed that the valinomycin-stimulated respiration of, and K^+ influx in, these bacteria were similar to the effect of this antibiotic on mitochondria. Associating this fact with the antibacterial action of valinomycin, they did not, however, carry out an experimental study of its antibacterial activity.

On the basis of available data, one can as yet arrive at no sufficiently clear understanding of the reason for such varied effects of valinomycin on K^+ transport in the different bacterial species. The object of the present study was to see whether such differences were associated with the nature of the energy supply for this process.

Three species of bacteria were investigated; *S. faecalis*, whose energy-linked ion transport proceeds only via degradation of glycolytic ATP, *M. lysodeikticus* which effects active ion transport via respiration and *Staph. aureus* whose energy-dependent ion transport is a function of both glycolytic ATP and respiration.

MATERIALS AND METHODS

Microorganisms. The antimicrobial potency of valinomycin relative to the K^+ and Na^+ contents of the medium was studied on the Gram-positive bacteria *Streptococcus faecalis*, *Staphylococcus aureus*, *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus subtilis*, the acid-fast bacterium *Mycobacterium phlei*, the Gram-negative bacterium *Escherichia coli* and the yeast *Candida albicans*. Valinomycin was prepared biosynthetically in the Institute [5].

Antimicrobial potency of valinomycin. The bacteriostatic effect of the antibiotic was determined by the serial tube dilution technique using a composite medium consisting of 10 g glucose, 5 g peptone, 30 ml of Hottinger's broth (750 mg % of amino-nitrogen) in 1 l of tap water (basic medium), pH 7.2, to which KCl and NaCl were added in various amounts. Ethanolic valinomycin solution was then added to the medium until a final concentration of 2% alcohol was obtained. Tubes with the same volume of ethanol but without the valinomycin were used as controls. The medium was inoculated with 1000 cells/ml of culture grown overnight on the basic medium, containing 80 mM NaCl. *Myc. phlei* inoculum was prepared by the method of Makeeva [6]. The test-tubes were incubated at 37 or 28 °C depending on the temperature optimum of the organism. Measurements were made 18 h after incubation, except for *Myc. phlei*, which was incubated for 3–4 days prior to measurement.

Effect of valinomycin on the intracellular K^+ and Na^+ contents of growing cultures. The bacteria were grown on the basic medium containing 80 mM sodium and 5 or 200 mM potassium. Bacterial growth was followed turbidimetrically at 600 nm. Valinomycin was added to the growing culture in the mid-exponential phase. Two hours later, intracellular K^+ and Na^+ were determined according to Epstein and Schultz [7]. The cells were filtered through 0.45 μm Millipore filters (Hufs) and washed with sucrose solution (0.25 M, 30 drops). The filters were then placed in polythene containers to which a few drops of concentrated HNO_3 was added. The residue was suspended in 5 ml 8 mM Li_2SO_4 and the K^+ and Na^+ concentrations were determined by flame photometry. The dry weights of the cells were calculated from the turbidimetric determination.

Preparation of resting cells for determination of energy-linked K^+ transport. We

selected three bacterial species differing in energy supply systems for the ion transport processes, namely, *Staph. aureus* 209P, *M. lysodeikticus Flemming* and *S. faecalis* BMK 6 (from the Institute of Microbiology USSR Ac. Sci). The bacteria were grown overnight on the above-described basic medium containing 80 mM NaCl. In order to avoid glycolysis *Staph. aureus* was cultivated on the same medium but without the glucose. The cells were collected by centrifugation, washed twice with 2 mM $MgSO_4$ and resuspended in potassium phosphate buffer of pH 7.2. For the partial depletion of the energy sources the bacteria were then incubated for 30 min at 37 °C, with shaking. Following this, the cells were harvested and washed twice with solution containing 1 mM KCl and 2 mM $MgSO_4$. Finally, a suspension containing 30–32 mg dry weight cells per ml of medium (0.5 M mannitol, 2 mM $MgSO_4$, 1 mM KCl) was made up.

Potassium transport. Potassium transport in the cells was studied by measuring the change in K^+ concentration of the incubation medium, using a K^+-sensitive glass electrode in a thermostated cuvette at 26 °C. The medium was maintained at constant pH by a pH-stat that automatically added HCl or Tris · OH as required. The proton transport was followed titrimetrically. The incubation mixture contained 0.5 M mannitol, 2 mM $MgSO_4$, 1 mM KCl and 10 mM glucose (as substrate), besides 10^{-6} M valinomycin, and 10^{-6} M *p*-trifluoromethoxyphenylhydrazone (FCCP) as proton conductor. The concentration of bacterial cells was 2.8 mg dry weight/ml. Aerobic or anaerobic conditions were provided by continuously bubbling air or nitrogen, respectively, through the medium.

Respiration of the bacterial cells. Respiration was measured polarographically using a platinated electrode. The incubation mixture containing 2.8 mg dry weight cells/ml consisted of 0.5 M mannitol, 0.05 M Tris · HCl (pH 7.5), 2 mM $MgSO_4$, 10 mM glucose.

RESULTS

Dependence of the bacteriostatic effect of valinomycin on the K^+ and Na^+ contents of the medium

The antimicrobial activity of valinomycin and its dependence on the K^+ and Na^+ content of the medium have already been touched upon in previous papers of this series [8–10]. In the present article the results are presented in greater detail (Table I). As one can see from Table I, the bacteria fall into three groups relative to their response to the addition of valinomycin at different K^+ concentrations: (1) loss of susceptibility to valinomycin at higher K^+ concentrations (*S. faecalis*); (2) the acquisition or increase of valinomycin susceptibility at higher K^+ concentrations (*Staph. aureus*, *M. lysodeikticus*, *Sa. lutea* and *B. subtilis*); (3) indifference to the K^+ content of the medium (*Myc. phlei* and *C. albicans*). Variations in the sodium content of the medium ranging from 3 to 200 mM sodium chloride (with the exception of *Sa. lutea* whose growth was no longer inhibited by valinomycin when the NaCl concentration became 150 mM) did not affect the antimicrobial activity of valinomycin. The lack of susceptibility of *E. coli* to the antibiotic seems to be due to a certain peculiarity in its cell wall composition, since it becomes quite susceptible after treatment with Tris/EDTA according to Harold [11].

Thus, the K^+ concentration in the medium is of major importance in manifes-

TABLE I

THE ANTIMICROBIAL ACTIVITY OF VALINOMYCIN AS A FUNCTION OF THE K^+ AND Na^+ CONTENT OF THE MEDIUM

The antimicrobial activity of valinomycin was determined by the serial tube dilution technique using a medium with varying amounts of K^+ and Na^+ at pH 7.2. An ethanolic valinomycin solution or ethanol alone was added to the medium until a final concentration of 2 % alcohol was obtained.

Microorganism	Minimal growth inhibiting valinomycin concentration ($\cdot 10^6$ M) in a medium containing:			
	5 mM K^+ 3 mM Na^+	200 mM K^+ 3 mM Na^+	5 mM K^+ 200 mM Na^+	200 mM K^+ 200 mM Na^+
Streptococcus faecalis	0.2	10	0.2	10
Staphylococcus aureus 209P	10	0.2	10	0.2
Micrococcus lysodeikticus	10	0.2	10	0.2
Sarcina lutea	0.1	0.01	0.1	0.01
Bacillus subtilis	10	1	10	1.5
Mycobacterium phlei	0.3	0.3	0.3	0.3
Candida albicans	0.2–0.4	0.2–0.4	0.2–0.4	0.2–0.4
Escherichia coli B	10	10	10	10

tation by valinomycin of an antimicrobial effect on *S. faecalis* and bacteria of the second group. By increasing the K^+ content of the medium we diminish its concentration gradient across the membrane. In the case of *S. faecalis*, valinomycin is effective provided that the K^+ gradient is sufficiently large. For bacteria of the second group, valinomycin-induced growth inhibition occurs only in the absence of a large K^+ gradient.

TABLE II

EFFECT OF VALINOMYCIN ON THE INTRACELLULAR ION CONTENT OF GROWING BACTERIAL CELLS

Ethanolic valinomycin 10^{-6} M or ethanol only (as control) was added to the growing cultures in the mid-exponential phase. Two hours later, intracellular K^+ and Na^+ were determined by flame photometry.

Microorganism	Ion	Ion content of cells growing on media (μM/g dry weight)			
		with 5 mM K^+		with 200 mM K^+	
		Control	Experiment	Control	Experiment
S. faecalis	K^+	650	50	670	500
	Na^+	100	310	60	90
Staph. aureus (medium with glucose)	K^+	740	520	700	700
	Na^+	70	110	70	70
Staph. aureus (medium without glucose)	K^+	620	960	1000	1620
	Na^+	170	240	180	450
M. lysodeikticus	K^+	730	1300	1200	1900
	Na^+	340	480	250	280

Effect of valinomycin on the ion content of growing cells

In order to understand the relation of the valinomycin activity to the K^+ concentration of the medium we carried out a series of experiments on growing cells of *S. faecalis*, *Staph. aureus* (with or without glucose in the medium) and *M. lysodeikticus*, measuring the bacterial growth and intracellular K^+ and Na^+ content in the presence and absence of the antibiotic. Some typical results are shown in Table II and Fig. 1. Two media were used, one containing 5 mM K^+ (Fig. 1A), the other 200 mM K^+ (Fig. 1B), the sodium concentration in both being 80 mM.

Valinomycin inhibited *S. feacalis* growth when the medium contained 5 mM K^+. The growth inhibition was accompanied by K^+ efflux from, and Na^+ entry into, the cells. In a medium containing 200 mM K^+ the decrease in intracellular K^+ on addition of valinomycin was insignificant. Culture growth was somewhat slower than in the control. These results are fully in agreement with Harold's data on annulation of the effect of valinomycin by excess K^+ [1].

Staph. aureus (in a non-glucose medium) and *M. lysodeikticus* were selected as

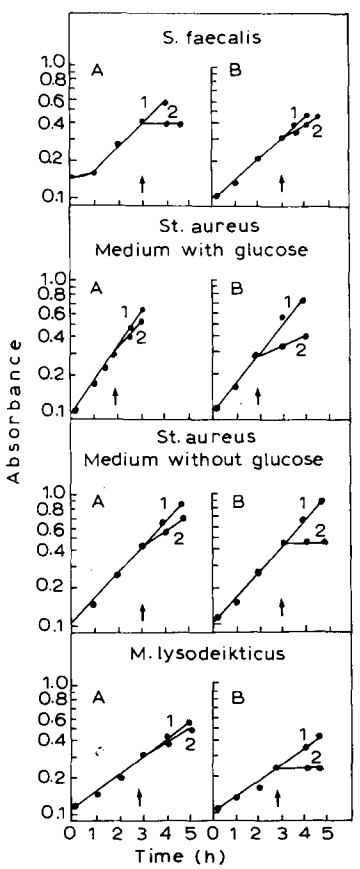

Fig. 1. Effect of valinomycin on bacterial growth. (1) Control, arrows indicate the time at which ethanol was added to a final concentration of 2 %. (2) As for (1), but with addition of valinomycin to 10^{-6} M. The medium contained (A) 5 mM K^+, (B) 200 mM K^+.

examples of respiring bacteria. Their K^+ content was dependent on the medium K^+ concentration. It was 1.5 times higher in cells grown on a medium with 200 mM K^+ than on a medium with 5 mM K^+. Addition of valinomycin increased the intracellular K^+ content in these bacteria, independent of the environmental concentration of this ion. The K^+ content of the cells growing in the presence of valinomycin in a 5 mM K^+ medium was similar to that for a 200 mM K^+ medium but without the antibiotic. One thus obtains the impression that when the amount of K^+ in the cells owing to the presence of valinomycin exceeds a certain limit, say 1500 mM/g dry weight, the growth rate of the bacteria abruptly falls. It is noteworthy that in the presence of antibiotic increase in the internal Na^+ was also observed as a rule. The reason for this is still obscure.

Similar experiments were carried out on *Staph. aureus* cells under aerobic conditions on a glucose-supplemented medium (the cells can effect both glycolysis and oxidative phosphorylation). The effect of valinomycin on the ion content and on the growth of bacteria was insignificant with a medium containing 5 mM K^+. When the medium contained 200 mM K^+ the antibiotic inhibited the bacterial growth without, however, affecting the internal ion content.

We have thus observed valinomycin inhibition of bacterial growth to be accompanied by the following processes: a passive K^+ efflux down the concentrational gradient in glycolysing cells; increase in the active K^+ influx against the K^+ gradient in respiring cells; constant K^+ content in cells with the mixed type of energetic metabolism. Non-variation of the K^+ content in this case is probably due to the effect of valinomycin on both processes, namely, on the passive K^+ efflux and active K^+ influx. Very likely the effect of valinomycin on the growth of *Staph. aureus* is connected with dissipation of energy by the induced K^+ fluxes.

The bacteriostatic effect of valinomycin as function of the K^+ concentration of the medium depends on the type of bacteria. In the case of glycolysing bacteria, inhibition occurs in the presence of a large concentration gradient, whereas, in the case of respiring bacteria, the gradient at which the antibiotic exerts its bacteriostatic effect must be much less.

The reason for inhibition of *Staph. aureus* growth (under conditions of mixed energetic metabolism) despite the non-changing ion content of the cells remains to be explained.

In the light of our results one might mention that in a study of the role of K^+ in acidic amino acid transport in the presence of valinomycin, the process is also dependent on the species of bacteria [12]. In *Staph. aureus* the inhibiting effect of valinomycin was observed only at high (50 mM) concentrations of K^+, whereas amino acid transport in *S. faecalis* is highly sensitive to valinomycin in the absence of added K^+.

Effect of valinomycin on energy-dependent K^+ transport

The experiments were performed on resting (non-growing) cells of three bacterial species with practically identical internal K^+ and internal Na^+ contents (Table III).

S. faecalis and *Staph. aureus* under anaerobic conditions were selected to typify the glycolysing bacteria. According to Harold [1] valinomycin affected K^+ transport in *S. faecalis* only at pH 6.0 and had no effect at pH 7.5. We also compared the effect of this antibiotic on the K^+ transport at low and high pH values (6.5 and 7.5). Fig. 2

TABLE III

INTRACELLULAR ION CONTENT (μM/g DRY WEIGHT) IN RESTING BACTERIAL CELLS

Cells grown overnight on a medium supplemented, or not, with glucose, were harvested and prepared for the energy-linked transport determination as indicated in the experimental section.

Microorganism	K^+	Na^+
S. faecalis	350–450	100–180
Staph. aureus (medium with glucose)	600–700	50– 70
Staph. aureus (medium without glucose)	600–700	50– 70
M. lysodeikticus	550–650	70–120

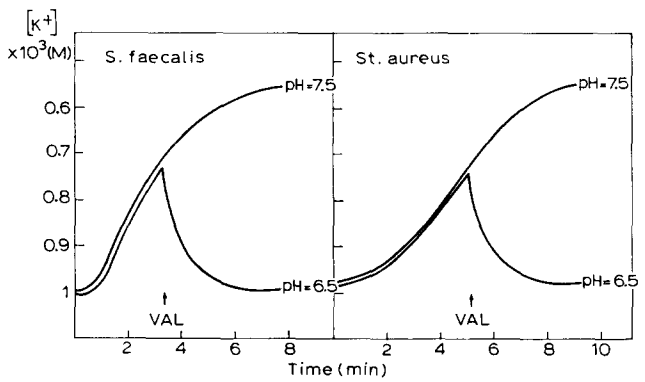

Fig. 2. Effect of valinomycin on energy-linked K^+ uptake by *Staph. aureus* (under anaerobic conditions) and *S. faecalis* cells. The incubation medium contained 0.5 M mannitol, 2 mM $MgSO_4$, 1 mM KCl, 2.8 mg dry weight cells/ml. Glucose (10 mM) was added at 0 min and valinomycin (10^{-6} M) at the time indicated by the arrow. The suspension was maintained at constant pH, under anaerobic conditions.

shows that the addition of glucose to the cells of both cultures induced the same rapid K^+ entry at both pH values. In agreement with Harold, our results show that valinomycin is inactive at pH 7.5. At pH 6.5 the antibiotic induced K^+ efflux down the concentration gradient from both *S. faecalis* and *Staph. aureus* cells. The K^+ outflow was accompanied by H^+ entry. The process was followed titrimetrically (results not shown). The simultaneous addition of valinomycin and the proton conductor FCCP resulted in still more rapid K^+ release by the cells.

Fig. 3 shows the effect of valinomycin on K^+ transport in respiring bacterial cells (*M. lysodeikticus* and *Staph. aureus* grown on a non-glucose-containing medium). The respiration rate was about 14 and 11 natom O/min per mg dry weight of cells for *M. lysodeikticus* and *Staph. aureus*, respectively. Valinomycin enhanced the oxygen consumption rate by 10–20%. Glucose induced K^+ influx into the cells against a concentration gradient, the process being considerably augmented by the addition of the antibiotic. Both the natural and induced K^+ flows were more intense at pH 7.5 than at 6.5. Subsequent addition of FCCP led to efflux of the K^+ down the concentration gradient in exchange for H^+ of the medium. Such passive K^+/H^+ exchange was more intense at pH 6.5.

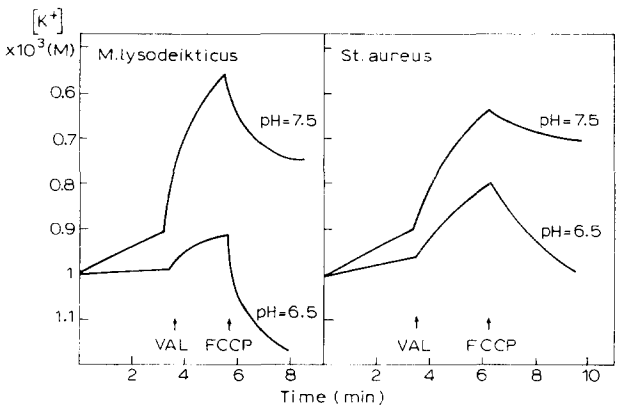

Fig. 3. Effect of valinomycin on the energy-linked K^+ uptake by *Staph. aureus* (grown under aerobic conditions in a glucose-free medium) and *M. lysodeikticus* cells. Glucose (10 mM) was added at 0 time, valinomycin (10^{-6} M) after 3.5 min and FCCP (10^{-6} M) after 6 min. The experiment was carried out under aerobic conditions at constant pH. Medium as described in Fig. 2.

Aerobic growth of *Staph. aureus* cells on a glucose-supplemented medium displayed a more complicated response to valinomycin addition. Without valinomycin the following processes could be observed: a fall in pH of the medium, augmentation of the cell respiration rate and K^+ influx into the cells. When valinomycin was added, the K^+ influx increased at pH 7.5, but fell at pH 6.5 (Fig. 4). We were probably recording the resultant of two processes, augmented K^+ entry due to activity of the respiratory chain (Fig. 3) and passive K^+ efflux caused by glycolysis (Fig. 2).

Thus, the effect of valinomycin on the energy-linked K^+ transport in resting cells is in complete harmony with its effect on the ion content of growing cells.

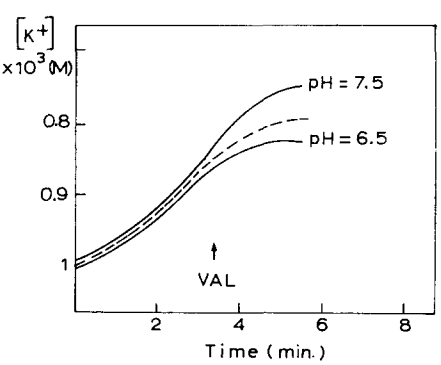

Fig. 4. Effect of valinomycin on the energy-linked K^+ uptake by *Staph. aureus* cells (grown under aerobic conditions on a glucose-supplemented medium). The conditions and the medium were as described in the legends to Fig. 3. Dotted line shows the rate of K^+ uptake when only ethanol (2 %) was added.

DISCUSSION

Our experiments have demonstrated that valinomycin acts differently on K^+ transport in glycolysing cells than in respiring cells under similar conditions. The addition of valinomycin to respiring cells enhances K^+ influx against the concentrational gradient in both growing and resting cells, whilst glycolysing cells treated with this antibiotic experience passive K^+ efflux down the concentration gradient. Valinomycin is known to induce K^+ flow down an electrochemical gradient. The results of our experiments lead us to the conclusion that there are different energy-dependent K^+ driving forces in glycolysing and respiring bacteria.

The rate increase in valinomycin-induced K^+ influx indirectly supports the suggestion that respiring bacteria develop a large metabolic electrical potential serving as driving force for the electrophoretic K^+ uptake in its presence. Indeed, a number of bacterial species (*M. denitrificans* [13], *Staph. aureus* [14], *E. coli* K_{12} [15]) are known to generate a membrane potential of about 200 mV (negative interior) under aerobic conditions. The data on respiring bacteria are in agreement with Mitchell's hypothesis that the metabolic potential could be the driving force for K^+ transport.

At the same time, the effect of valinomycin on glycolysing cells suggests that these bacteria, unlike respiring cells, do not generate a membrane potential sufficient to cause K^+ flux against the concentration gradient. This is in complete agreement with Harold and Papineau [16] who demonstrated that *S. faecalis* cells replete with K^+ apparently do not maintain a large potential. Harold concluded that the electrical potential is not sufficient to account for the active K^+ transport [3].

It is obvious that glycolysing *S. faecalis* and *Staph. aureus* cells (under anaerobic conditions) accumulate large amounts of K^+ against its gradient. Unlike transport in respiring cells, the energy-linked K^+ transport in glycolysing cells is unaccompanied by the generation of significant potentials across the membrane. The mechanism of the active K^+ transport is as yet obscure. As far as we know, no data have been published concerning the effect of valinomycin on the processes of active K^+ transport inherent in the bacterial cell. The valinomycin effect is nullified by addition of KCl or by raising the pH. This suggests that, in itself, valinomycin has no effect on the energy-dependent K^+ transport in glycolysing cells. Evidently it enhances the K^+ permeability of the membranes and thereby causes K^+ efflux down the concentrational gradient. Addition of the proton conductor FCCP accelerates this efflux.

The results obtained with *S. faecalis* cells were similar to those obtained with *Staph. aureus* cells (under anaerobic conditions). Moreover, valinomycin was observed to have a similar effect on the K^+ flux in *E. coli* K_{12} cells, which like *Staph. aureus* are characterized by a mixed type of energetic metabolism. The antibiotic induces passive K^+ efflux under anaerobic conditions and an increase in K^+ influx under aerobic conditions. One might reasonably conclude that the effects demonstrated on *S. faecalis* could be extended to other glycolysing bacteria in which the ion transport is dependent on the ATPase activity.

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