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Cation Transport and Electrogenesis by *Streptococcus faecalis*

I. The Membrane Potential

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Summary. Uptake of the lipid-soluble cations dibenzyldimethylammonium (DDA^+) and triphenylmethylphosphonium ($TPMP^+$) by *Streptococcus faecalis* is biphasic. The initial phase is a rapid binding of the ions which does not require a source of metabolic energy and apparently consists of cation exchange at the cell surface. Upon addition of glucose further uptake of the cations occurs, by exchange for Na^+ and H^+. Evidence is presented suggesting that this metabolic uptake of DDA^+ and $TPMP^+$ is not due to active transport. It rather appears that uptake results from the generation of an electrical potential, interior negative, by the extrusion of H^+ and, indirectly, of Na^+. Accumulated DDA^+ and $TPMP^+$ are discharged by proton-conducting uncouplers. The cation-conducting antibiotics valinomycin, monactin, nigericin and monensin do not inhibit uptake. Potassium and, under certain conditions, H^+ displace DDA^+ and $TPMP^+$. Generation of an electrical difference across the membrane was verified by the accumulation of K^+ in the presence of valinomycin. The concentration ratios achieved correspond to potentials of the order of -150 to -200 mV.

Potassium is the major intracellular cation of growing microbial cells, even when Na^+ predominates in the external environment. However, cells harvested from the stationary phase of growth are often partially depleted of K^+ and contain Na^+ and H^+ instead. When such cells are transferred to a medium containing K^+ and a source of energy they quickly reaccumulate K^+ with concurrent extrusion of Na^+ and H^+. *Streptococcus faecalis* has proven to be convenient for studies on energy-linked cation transport: The organism lacks internal energy reserves, and K^+ uptake is therefore strictly dependent upon provision of an exogenous energy source. Moreover, *S. faecalis* lacks cytochromes and carries out little, if any, oxidative phosphorylation. The cells derive metabolic energy from glycolysis or arginine degradation, and it is almost certain that adenosine triphosphate (ATP) is the ultimate energy donor for cation transport. The ATPase

associated with the cytoplasmic membrane is believed to couple the metabolism of ATP to cation transport, but the precise function of this enzyme is not known (Deibel, 1964; Zarlengo & Schultz, 1966; Harold & Baarda, 1968*a*; Harold, Baarda, Baron & Abrams, 1969; Schnebli, Vatter & Abrams, 1970).

A number of mechanisms can be envisaged to account for the coupling of net K^+ uptake to net extrusion of Na^+ and H^+. Among these are obligatory coupling of K^+ and Na^+ movements at the level of the ATPase, as is the case in mammalian cells; an electrogenic, inward-directed K^+ pump; primary pumps moving H^+ and Na^+ outwards; and other mechanisms. Our earlier attempts to distinguish among the various possibilities (Harold, Baarda & Pavlasova, 1970*a*; Harold, Pavlasova & Baarda, 1970*b*) led to an interpretation derived from the chemiosmotic hypothesis (Mitchell, 1966, 1970): The primary process would be the extrusion of H^+ and perhaps of Na^+, from the cells. This generates an electrical difference across the membrane, interior negative; K^+ accumulation would occur in response to this electrical gradient.

Critical examination of this hypothesis requires the demonstration that metabolizing cells do indeed generate the predicted electrical difference across the membrane. In the absence of microelectrodes small enough for use with bacteria, we have adopted the approach pioneered by investigators in the U.S.S.R. (Liberman & Topaly, 1968; Liberman, Topaly, Tsofina, Jasaitis & Skulachev, 1969; Grinius, Jasaitis, Kadziauskas, Liberman, Skulachev, Topali, Tsofina & Vladimirova, 1970; Bakeeva, Grinius, Jasaitis, Kuliene, Levitsky, Liberman, Severina & Skulachev, 1970; Liberman & Skulachev, 1970; *see also* Mitchell & Moyle, 1969). Briefly, ions which diffuse passively across the membrane should be distributed between cells and medium in accordance with the membrane potential. Assuming ideal behavior, the membrane potential can be estimated from the Nernst equation,

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[\text{cation}]_o}{[\text{cation}]_i} \times \frac{f_o}{f_i}$$

where f is the activity coefficient, i and o refer to the internal and external phases, respectively, $\Delta\Psi$ is the electrical potential and R, T and F have their usual meanings. A lucid review summarizing earlier applications of electrochemical principles to bacteria has been prepared by Cirillo (1966).

We report here studies on the uptake of the lipid-soluble cations dibenzyldimethylammonium (DDA^+) and triphenylmethylphosphonium ($TPMP^+$), whose structures are shown in Fig. 1. The results strongly support the

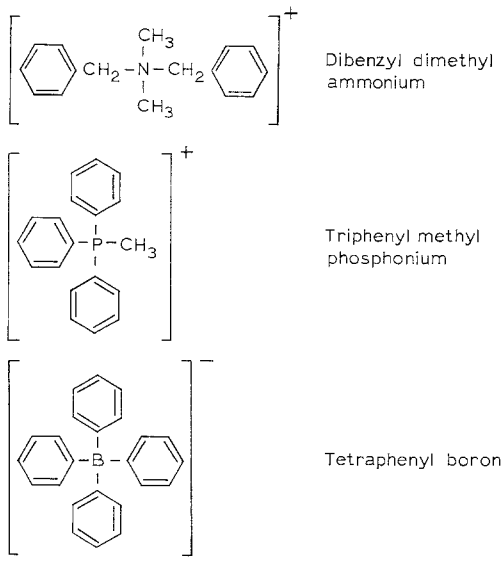

Fig. 1. Chemical structures of the lipid-soluble cations and anions

proposal that glycolyzing cells extrude H^+, and indirectly Na^+ as well, but do not extrude K^+. An electrical potential is thus generated, of the order of -200 mV, interior negative. The origin of this potential and its role in K^+ uptake will be considered in the following paper.

Materials and Methods

Cell Preparations

Streptococcus faecalis strain 9790 was grown on the following complex media: Medium NaTY, containing 1% Na_2HPO_4, 1% bacto tryptone, 1% glucose and 0.5% yeast extract (this medium is approximately 0.15 M Na^+ and 5 mM K^+); medium KTY contains K_2HPO_4 in place of Na_2HPO_4.

Four distinct cell preparations were employed in this and the following study:

(1) *KTY Cells.* Organisms were grown overnight on medium KTY. The suspension was neutralized; the cells were then centrifuged and washed twice with 2 mM $MgSO_4$ before use. Typically such cells contain 1,000 to 1,200 μmoles K^+ per g dry weight.

(2) *NaTY Cells.* Organisms were grown overnight on medium NaTY, harvested and washed with 2 mM $MgSO_4$. Cells grown in this medium are partly depleted of K^+: A typical batch contains Na^+ and K^+, each at about 500 μmoles/g dry weight. In addition, the cells have considerable amounts of H^+ such that the cytoplasmic pH is as low as 5 (Zarlengo & Abrams, 1963; Zarlengo & Schultz, 1966).

(3) *Na^+-Loaded Cells.* To replace K^+ completely by Na^+, cells of either of the above types were incubated with the antibiotic monactin in 0.1 M sodium maleate, pH 8. After 20 min the antibiotic was removed by repeated washing of the cells with buffer and water (Harold & Baarda, 1968*a*).

(4) *H^+-Loaded Cells.* Partial replacement of K^+ by H^+ was carried out by a similar procedure. KTY cells were suspended in water and incubated with monactin at room temperature, while the pH was kept at 6.0 by addition of HCl (Harold & Baarda, 1968*a*).

General Experimental Conditions

All cell preparations were finally resuspended in water, generally at a density of 1 to 2 mg cells (dry weight) per ml. It should be stressed that *S. faecalis* is highly resistant to osmotic shock, and does not release metabolites even when suspended in water. Experiments were conducted at room temperature, with arginine (0.01 M) or glucose (0.01 M) as energy source. The rate of glycolysis was monitored at constant pH by automatic titration of the lactic acid produced, by use of a Radiometer pH-Stat (Tris or NaOH in the reservoir). At intervals, samples were quickly filtered through Millipore filters (0.45 μ) and the filtrate or cells were analyzed.

Analytical Methods

Procedures for determination of K^+ and Na^+ by flame photometry have been described earlier (Harold & Baarda, 1967; Harold *et al.*, 1969).

Dibenzyldimethylammonium ion (DDA^+) and triphenylmethylphosphonium ion ($TPMP^+$) were determined in filtrates by means of their ultraviolet absorption spectra, shown in Fig. 2. Solutions and filtrates were treated with an anion exchange resin (Whatman DE52, microgranular, free base; 100 mg/ml) to adsorb interfering substances such as nucleotides; the resin was then removed by filtration through Pyrex fiberglass wool, and the entire absorption spectrum of each sample was determined. For DDA^+, the extinction coefficient at 262 nm is 830 liters/mole cm, and the ratio of optical densities at the peak and the neighboring trough, $\frac{\text{O.D. 262 nm}}{\text{O.D. 266 nm}} = 1.92$. Similarly, for $TPMP^+$, the extinction coefficient at 266.5 nm is 3,100 liters/mole cm, and the ratio $\frac{\text{O.D. 266.5 nm}}{\text{O.D. 270.5 nm}}$

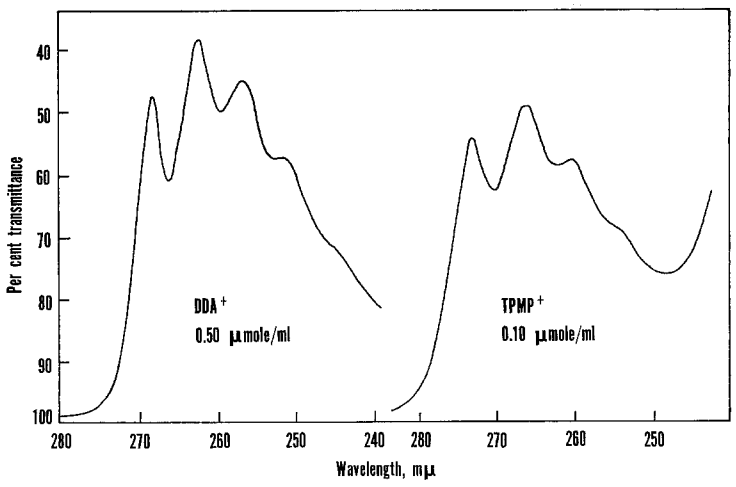

Fig. 2. Absorption spectra of DDA^+ and $TPMP^+$ (chloride and bromide salt, respectively, in aqueous solution)

is 1.54. To calculate the concentration of the cations in filtrates, a baseline was subtracted from the absorbance values for peak and trough so as to bring the ratio of the two into accord with that of pure samples. By this procedure, DDA^+ could be estimated to concentrations as low as 0.05 μmoles/ml, and $TPMP^+$ down to 0.02 μmoles/ml.

The concentration of cations in the cellular water space was calculated from the amount removed from the medium. The water space of *S. faecalis* was taken to be 2.43 ml/g (dry weight) of cells (Harold *et al.*, 1970*b*).

Chemicals

Dibenzyldimethylammonium chloride (DDA^+), triphenylmethylphosphonium bromide ($TPMP^+$), tetrabutylammonium bromide and tetraphenylboron (TPB^-) were purchased from K & K Laboratories. They were dried over P_2O_5; recrystallization of the commercial materials did not alter their absorption spectra. The sources of antibiotics and other inhibitors will be found in earlier publications.

Results

Accumulation of DDA^+ by Sodium-Loaded Cells

The basic observations which form the point of departure for subsequent experiments are shown in Fig. 3. Uptake of DDA^+ by sodium-

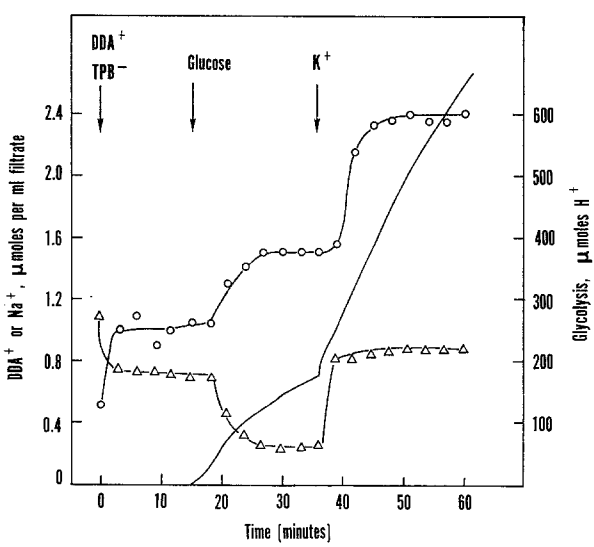

Fig. 3. Uptake of DDA^+ by sodium-loaded cells. A suspension of sodium-loaded cells in water (100 ml, 2 mg cells per ml) was kept at pH 7.5. At 0 min, DDA^+ (1.0 μmoles/ml) and TPB^- (0.01 μmoles/ml) were added. Glucose (0.01 M) was added at 15 min, K^+ (3 μmoles/ml) at 36 min. Samples of medium and cells were collected at intervals for determination of DDA^+ and Na^+. The plot shows removal of DDA^+ from the medium and the appearance of Na^+ in its place. ▵, DDA^+; ○, Na^+; ———, glycolysis (cumulative)

loaded cells of *S. faecalis* consisted of two phases: A rapid uptake which was independent of metabolism; and a slower, but more extensive uptake initiated upon addition of a source of metabolic energy. These observations parallel the findings of Bakeeva *et al.* (1970) with mitochondria and, on the assumption that DDA^+ is not accumulated by active transport, constitute *prima facie* evidence for the generation of an electrical potential, interior negative, during glycolysis. It is convenient to consider first the two phases of DDA^+ uptake. The subsequent displacement of DDA^+ by K^+ will be discussed in a later section of this paper.

(1) Addition of DDA^+ to a suspension of cells, in the absence of glucose, resulted in rapid binding of the DDA^+ by the cells. This nonmetabolic uptake was abolished by 2 mM Mg^{++}, 2 mM Ca^{++} and by 1 mM spermine. K^+, Na^+ and Li^+ at 10 mM reduced the nonmetabolic uptake to one-half or less of the amount removed from water (data not shown). In the absence of glucose, neither the monovalent nor the divalent cations readily pass across the membrane. In the particular experiment illustrated in Fig. 3, the lipid-soluble anion TPB^- was also added, for reasons to be stated shortly. However, the extent of nonmetabolic uptake of DDA^+ was the same whether TPB^- was present or not (data not shown). Finally, uptake of DDA^+ under these conditions was not inhibited by proton conductors. Taken together, these observations make it likely that nonmetabolic uptake is a process of ion exchange at the cell surface, and does not involve passage across the osmotic barrier.

(2) The main subject of this paper is the second phase of DDA^+ uptake, which is initiated by the addition of glucose. As had previously been found for mitochondria (Bakeeva *et al.*, 1970), the rate of DDA^+ uptake by glycolyzing cells was greatly enhanced by catalytic concentrations of tetraphenylboron (TPB^-), a lipid-soluble anion. For this reason TPB^- was added together with DDA^+ in the experiment shown in Fig. 3 and in all subsequent ones.

TPB^- is not entirely innocuous for *S. faecalis*. At a concentration of 0.1 mM, TPB^- by itself inhibited growth and induced passive exchange of K^+ for Rb^+ or Na^+ across the membrane, presumably because of the formation of a lipid-soluble complex of TPB^- and K^+ (Mueller & Rudin, 1970). However, lower levels of TPB^- did not inhibit growth and appeared to have no adverse effects on the cells. In all the experiments reported here, TPB^- was employed at 0.01 mM. In addition, all conclusions were verified with $TPMP^+$ whose uptake does not require TPB^- (*see below*). The role of the anion is uncertain, but it most probably serves as a carrier for the cationic molecule (Bakeeva *et al.*, 1970); this assumption receives support

from the observation (data not shown) that TPB^- enhanced the rate, but not the extent, of DDA^+ uptake.

Accumulation of DDA^+ was accompanied by extrusion of an equivalent amount of Na^+. In the experiment shown in Fig. 3, the cells lost 225 μmoles Na^+ per g cells, while 235 μmoles DDA^+ were taken up. If it is assumed that all the DDA^+ taken up after addition of glucose is in free solution in the cytoplasm, its internal concentration would be 97 mM, while the external concentration is 0.23 mM. The concentration ratio of 420 would be in equilibrium with a Nernst potential of −155 mV, interior negative.

In most of our experiments glucose served as the energy source, but arginine catabolism also supported uptake of DDA^+. In one particular experiment DDA^+ accumulated in the cells to a concentration of 95 mM, while the external concentration was 0.44 mM. In general, however, arginine was considerably less effective than glucose as an energy source for the uptake of DDA^+.

Many of our experiments were performed with sodium-loaded cells, prepared by the use of the antibiotic monactin as described in Materials and Methods. Uptake of DDA^+ was also observed with NaTY cells, as will be described in the following report (Harold & Papineau, 1972). Uptake was neither dependent upon, nor inhibited by, prior exposure of the cells to monactin.

Accumulation of Other Lipid-Soluble Cations by Sodium-Loaded Cells

All the experiments described above were performed also with $TPMP^+$. The characteristics of $TPMP^+$ accumulation were qualitatively and quantitatively similar to those described for DDA^+: limited nonmetabolic uptake, followed by additional uptake when glucose was added. The only significant difference was that glycolyzing, sodium-loaded cells rapidly accumulated $TPMP^+$ even in the *absence* of TPB^-. Addition of the anion did not significantly enhance either the rate or the extent of $TPMP^+$ uptake. Some quantitative results will be presented below (Table 1).

Tetrabutylammonium ion could not be studied quantitatively since it lacks absorption in the ultraviolet, but measurements of sodium displacement indicate that this ion is also accumulated by glycolyzing cells.

K^+-Loaded Cells do Not Accumulate DDA^+

In contrast to the sodium-loaded cells discussed thus far, cells replete with K^+ did not exhibit any metabolic uptake of DDA^+ and $TPMP^+$.

Table 1. Distribution of DDA^+ and $TPMP^+$ between cells and medium as a function of the concentration of cation[a]

Cation	Concentration of cation (μmoles/ml)			
	Initial	Final, in medium	Final, in cells	$\frac{[\text{cation}]_i}{[\text{cation}]_o}$
DDA^+	0.1	0.04	13	325
	0.2	0.07	27	385
	1.0	0.34	138	405
$TPMP^+$	0.05	0.02	6	300
	0.18	0.05	27	540
	0.5	0.13	75	580
	1.0	0.30	145	485

[a] Sodium-loaded cells were suspended in 10 mM Na_2SO_4 at a density of 2 mg/ml. The pH was kept at 7.5 by means of the pH-stat. DDA^+ (together with 0.01 μmole/ml TPB^-) or $TPMP^+$ were added to the initial concentration listed. The cells were allowed to glycolyze and samples were withdrawn at intervals, filtered and analyzed as described in Materials and Methods. The internal concentrations are not corrected for the fraction of uptake that is independent of metabolism. Data reported refer to the distribution in the steady state, 20 min after addition of glucose.

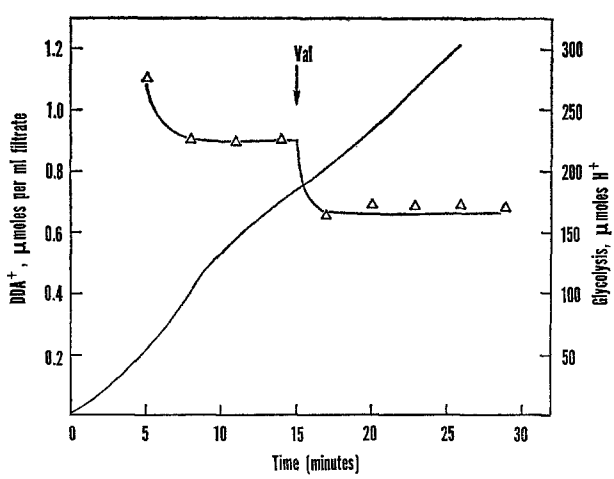

Fig. 4. K^+-loaded cells do not accumulate DDA^+. A suspension of KTY cells in water (2 mg cells per ml, 50 ml) was allowed to glycolyze at pH 7.5. At 5 min, DDA^+ (1.1 μmoles/ml) and TPB^- (0.01 μmole/ml) were added. Valinomycin (0.5 μg/ml) was added at 15 min. ▵, DDA^+; ———, glycolysis (cumulative). The plot shows removal of DDA^+ from the medium

Like the other preparations, cells grown on KTY medium accumulated small amounts of DDA^+ and $TPMP^+$ by ion exchange at the surface, but addition of glucose elicited no additional uptake. Only in the presence of valinomycin did we observe significant uptake of DDA^+, presumably because this antibiotic supplies a passive channel for K^+ to leave the cells (Fig. 4). (High concentrations of DDA^+, 5 mM or above, did displace K^+ even from normal cells but the physiological significance of this displacement is debatable.)

Validation of the Use of DDA^+ and $TPMP^+$ as Indicators of a Membrane Potential

Inference of a membrane potential from the distribution of an ion between the cell interior and the medium rests upon a number of assumptions. The purpose of this section is to make these explicit and to consider the justification and limitations of the procedures we have employed.

(1) DDA^+ and $TPMP^+$ are not chemically altered during uptake. Cells were allowed to accumulate DDA^+ or $TPMP^+$, filtered and the accumulated ions were then released from the cells by exchange for H^+ (*see below*). The DDA^+ or $TPMP^+$ thus recovered were found to be chemically identical with the starting material as judged by thin-layer chromatography and by their ultraviolet and infrared absorption spectra.

(2) DDA^+ and $TPMP^+$ do not seriously interfere with the integrity or metabolism of *S. faecalis*. At concentrations of 1.0 mM or less the substances did not inhibit growth in either KTY or NaTY medium; higher concentrations were inhibitory. It will be noted, however, that DDA^+ did reduce the rate of glycolysis somewhat (e.g., Fig. 5); $TPMP^+$ was even more inhibitory. The inhibition of glycolysis was reversed by K^+ (Fig. 3). Since neither phosphate nor nucleotides were lost from the cells, the integrity of the cell membrane appears to be unimpaired.

(3) DDA^+ and $TPMP^+$ diffuse passively across biological membranes in response to an electrical potential. Evidence that *S. faecalis can* accumulate these cations when an electrical potential is artificially applied across its cytoplasmic membrane is presented in Fig. 5: K^+-loaded cells, in the absence of an energy source, took up very little DDA^+ (or $TPMP^+$) but addition of valinomycin elicited immediate efflux of K^+ and equivalent uptake of DDA^+. Since valinomycin serves as a carrier for K^+, efflux of K^+ from the cells down the concentration gradient should generate a membrane potential, interior negative. At equilibrium the ratio of the concentrations

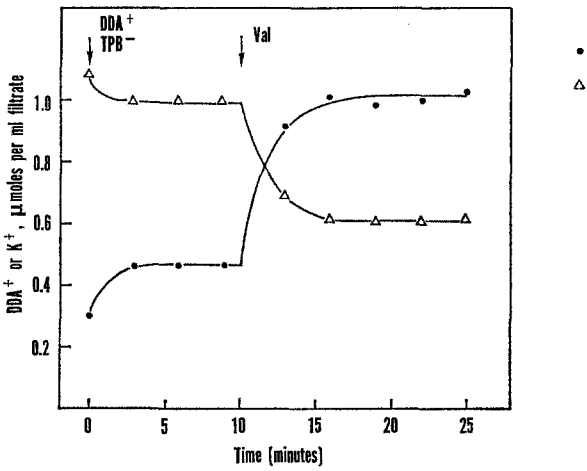

Fig. 5. Accumulation of DDA^+ by resting cells in response to an electrical potential. KTY cells were suspended in water (2 mg cells per ml, no glucose) and kept at pH 7.5. At 0 min, DDA^+ (1.0 μmole/ml) and TPB^- (0.01 μmole/ml) were added. Valinomycin (0.5 μg/ml) was added at 10 min. △, DDA^+; •, K^+

of the cation inside the cells and in the external medium should be the same; indeed, the ratios are found to be 140 for K^+, 137 for DDA^+.

Gramicidin was used in the same way to generate an artificial membrane potential and DDA^+ uptake in sodium-loaded cells. By contrast the antibiotics nigericin and monensin, which induce electrically neutral K^+/H^+ and Na^+/H^+ exchanges, did not elicit DDA^+ uptake.

(4) Accumulation of DDA^+ and $TPMP^+$ is not due to active transport. If active transport were responsible for the accumulation of DDA^+ and $TPMP^+$, one would expect to find evidence for binding of the cations to a carrier site. Thus the rate and extent of uptake would probably obey Michaelis-Menten kinetics. In an attempt to detect this, the accumulation of DDA^+ and $TPMP^+$ was studied over the range of concentrations from 0.05 to 1.0 μmoles/ml (the lower limit is set by the sensitivity of the analytical procedure, the upper by the capacity of the cells to accumulate cations). Table 1 lists the final distribution of DDA^+ and $TPMP^+$ between cells and medium; in each case this was attained within 20 min of the addition of glucose. We interpret these data to mean that the distribution of DDA^+ and $TPMP^+$ was approximately the same for both substances and independent of the external concentration added. The rates of uptake (data not shown) were also similar and independent of the external concentration. We must qualify this conclusion by admitting that the analytical method becomes

quite unreliable at the lower limits of the concentration range; these are, of course, precisely the points of greatest interest.

We conclude that no evidence was obtained either for saturation kinetics of uptake or for structural specificity. This argues against interaction of the cations with a rate-limiting carrier, but is not sufficient to rule out conclusively the existence of such a hypothetical carrier.

Effect of Ionophores on the Metabolic Accumulation of DDA^+

Experiments with ion-conducting agents provided further evidence that the accumulation of DDA^+ and $TPMP^+$ occurred in response to an electrical difference generated by the extrusion of cations from glycolyzing cells. The characteristics of these compounds have been reviewed (Pressman, 1968; Mueller & Rudin, 1970; Harold, 1970).

(1) Proton-conducting uncouplers, including tetrachlorosalicylanilide (TCS), carbonylcyanide-m-chlorophenyl hydrazone (CCCP) and tetramethyldipicrylamine (TMPA) completely blocked uptake of DDA^+ and $TPMP^+$, while glycolysis was unaffected or even accelerated. As illustrated in Fig. 6, addition of TCS to cells that had been first allowed to accumulate DDA^+ elicited immediate discharge of the cation. Glycolysis ceased transiently, but this effect is only apparent: it should be recalled that we

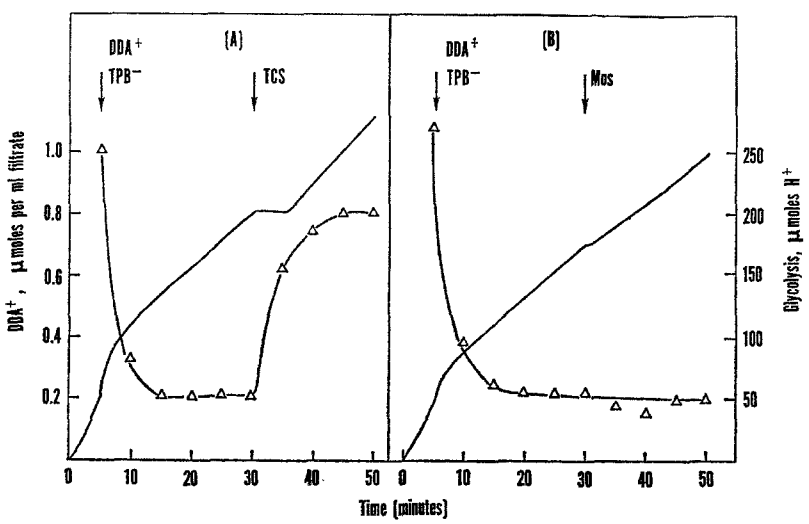

Fig. 6. Effect of ionophores on DDA^+ uptake. Sodium-loaded cells were suspended in water (2 mg cells per ml, 80 ml) and allowed to glycolyze at pH 7.5. At 5 min, DDA^+ (1.0 μmole/ml) and TPB^- (0.01 μmole/ml) were added. Suspension (A) received TCS, 2 μg/ml, at 30 min. Suspension (B) received monensin (Mos), 2 μg/ml, at 30 min. ▵, DDA^+; ———, glycolysis (cumulative)

measure glycolysis as H^+ production; this is interrupted while DDA^+ flows out, H^+ being retained by the cells to preserve electroneutrality. Glycolysis then resumed at an accelerated rate.

(2) The antibiotic nigericin catalyzes electrically neutral exchange of K^+ for H^+, whereas monensin exchanges Na^+ for H^+. Fig. 6 documents the fact that monensin did not inhibit uptake of DDA^+ by sodium-loaded cells. Similar results were obtained with nigericin, and neither antibiotic inhibited uptake of $TPMP^+$ (data not shown).

Monactin and valinomycin, antibiotics which facilitate K^+ diffusion with a very high degree of specificity, had no effect on sodium-loaded cells unless K^+ was also present. We shall return to the uptake of K^+ later in this paper and also in the following one.

Effect of H^+ and K^+ on DDA^+ Accumulation

The apparent potential difference maintained by glycolyzing cells should be collapsed by high concentrations of all permeant cations. Thus one may expect DDA^+ and $TPMP^+$ to be displaced by other cations; the effect of TCS, facilitating H^+ movements at pH 7.5, is a case in point.

Even in the absence of a proton conductor, H^+ proved to be a strong inhibitor of DDA^+ uptake: Sodium-loaded cells took up very little DDA^+ or $TPMP^+$ at pH 6.0. Cells which had previously accumulated DDA^+ at pH 7.5 released it when the pH was lowered to 6.0 (Fig. 7). The reason

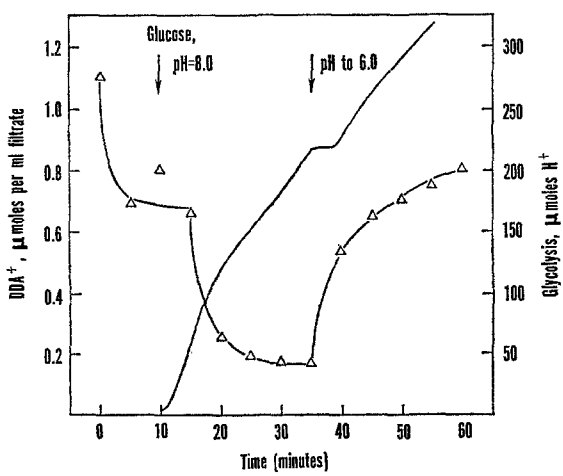

Fig. 7. Effect of pH on the uptake of DDA^+. Sodium-loaded cells were suspended in water (2 mg cells per ml, 80 ml). At 0 min, DDA^+ (1.1 µmoles/ml) and TPB^- (0.01 µmole/ml) were added. Glucose was added at 10 min and the cells were allowed to glycolyze at pH 8.0. At 35 min the pH was lowered to 6.0 by addition of HCl. ▵, DDA^+; ———, glycolysis (cumulative)

for this is far from clear. We can only suggest that at pH 6.0 protons pass across the membrane at a rate (Harold & Baarda, 1968*b*) sufficient to restrict the potential and thus limit uptake of the lipid-soluble cations.

Equally dramatic was the effect of K^+. At pH 7.5, 3.0 mM K^+ displaced DDA^+ almost instantaneously (Fig. 3), whereas Na^+ had very little effect even at 50 mM (not shown).

Estimation of the Electrical Potential from K^+ Uptake in the Presence of Valinomycin

Valinomycin facilitates the diffusion of K^+ in the direction of its electrochemical gradient across many biological and artificial membranes (Reviews: Pressman, 1968; Mueller & Rudin, 1970; Harold, 1970). Evidence that the

Table 2. Estimation of the electrical potential from the uptake of K^+ in presence of valinomycin[a]

Additions	Initial concentration added to medium (μmoles/ml)		Final concentrations in medium and cells (μmoles/ml)					
	K^+	DDA^+	K_o^+	K_i^+	K_i^+/K_o^+	DDA_o^+	DDA_i^+	DDA_i^+/DDA_o^+
K^+ only	1.0	—	0.10	185	1850		—	
DDA^+, TPB^-	—	1.0		—		0.25	155	620
K^+ plus DDA^+, TPB^-	0.40	0.40	0.035	74	2100	0.10	62	620

[a] Sodium-loaded cells were suspended in water at 2 mg/ml and allowed to glycolyze at pH 7.5. Valinomycin was added to 0.5 μg/ml. After 5 min, K^+, DDA^+ or both were added as shown. Samples were filtered and analyzed at intervals; the steady state was attained within 5 min for K^+, after 10 to 15 min for DDA^+. The internal concentrations are not corrected for the fraction of uptake that is independent of metabolism.

antibiotic catalyzes rapid diffusion of K^+ across the membrane of *S. faecalis* was presented in an earlier paper (Harold & Baarda, 1967). Therefore, the extent of K^+ accumulation in the presence of the antibiotic can be used to provide an independent estimate of the membrane potential, as described by Mitchell & Moyle (1969). Measurements on K^+ accumulation by sodium-loaded cells of *S. faecalis* are summarized in Table 2. The results indicate that the internal K^+ concentration is about 2,000 times higher than the external one, corresponding to a potential of -195 mV. K^+ accumulation

is clearly more efficient than that of DDA^+. The disparity between the uptake of K^+ and that of the lipid-soluble cations persists even when uptake of both is measured simultaneously (Table 2).

Discussion

The basic observation reported here is that cells of *S. faecalis* accumulate large quantities of DDA^+, $TPMP^+$ and other lipid-soluble cations by exchange for Na^+. Part of this uptake occurs even in resting cells and is apparently due to ion exchange at the cell surface. Of greater interest is the second phase of uptake which requires either glucose or arginine and results in the accumulation of up to 0.1 M DDA^+ or $TPMP^+$ in the cytoplasm, with a concentration gradient of about 500 (Fig. 3). It is our contention that glycolyzing cells extrude H^+ and Na^+, but not K^+, and thereby generate an electrical potential across the membrane (interior negative). Accumulation of the lipid-soluble cations would be a thermodynamically passive response to the potential gradient.

The conclusion, that DDA^+ and $TPMP^+$ are not actively transported against the electrochemical gradient but move in response to an electrical potential, rests on the following arguments.

(1) DDA^+ and $TPMP^+$ extracted or released from the cells are indistinguishable from the starting material. There is nothing to suggest that uptake of the cations occurs by group translocation.

(2) The existence of a carrier which recognizes the unnatural and structurally diverse ions DDA^+, $TPMP^+$ and tetrabutylammonium is inherently improbable. Stimulation of DDA^+ uptake by tetraphenylboron also suggests that for this cation, at least, there is no natural carrier. Finally, our failure to detect saturation of rate and pool size argues against interaction with a rate-limiting carrier.

(3) The induction of DDA^+ and $TPMP^+$ uptake in resting cells by addition of valinomycin (Fig. 5) is evidence that these cations do diffuse passively across the cytoplasmic membrane of *S. faecalis* in response to an electrical potential. Passive diffusion of these ions across artificial phospholipid bilayers was demonstrated earlier by Liberman *et al.* (1969) and by Grinius *et al.* (1970).

(4) DDA^+ and $TPMP^+$ are discharged from glycolyzing cells by TCS and CCCP (Fig. 6). These compounds are known to mediate the electrogenic passage of protons across the plasma membrane, and would collapse the electrical potential by permitting glycolytic H^+ to flow back into the cells.

By contrast, the antibiotics nigericin and monensin, which catalyze electroneutral exchange of K^+ or Na^+ for H^+, neither inhibit uptake of DDA^+ and $TPMP^+$ nor elicit their release (Fig. 6). Thus, it is not proton entry *per se*, but collapse of the potential, which accounts for the discharge of DDA^+.

(5) Independent evidence for the generation of an electrical potential by glycolyzing cells comes from the finding that such cells continue to accumulate K^+ even in the presence of valinomycin (Harold *et al.*, 1970*a* and Table 2). This antibiotic, by forming a lipid-soluble complex with K^+, in effect renders the cytoplasmic membrane freely permeable to K^+. Distribution of K^+ between cells and medium should therefore reflect the electrical potential (Mitchell & Moyle, 1969).

No single one of these arguments is conclusive by itself, but taken together they render active transport of DDA^+ and $TPMP^+$ most unlikely. All the evidence is, however, consistent with the hypothesis that sodium-loaded cells generate a substantial electrical potential during glycolysis.

The magnitude of the potential can be estimated from the distribution of DDA^+, $TPMP^+$ and K^+, though the assumptions required render the calculation approximate at best. The conventional assumption that the cell interior is a single compartment of constant volume is fairly safe, though the use of inulin (Harold *et al.*, 1970*b*) may have led us to overestimate the water space. Somewhat more questionable is the assumption that the cell wall makes a negligible contribution to the permeability barrier for such bulky ions as DDA^+ and $TPMP^+$. This assumption is implicit in the calculation of the potential by the Nernst equation, since it is tacitly assumed that concentration of the cations in the medium equals that at the membrane surface. Slayman (1965) has explicitly considered the contribution of the cell wall to the electrical potential across the surface layers of *Neurospora*. The least reliable postulate is surely that DDA^+ and $TPMP^+$ exist within the cell in free solution. The fractions associated with membrane, wall or ribosomes are unknown, and the activity coefficient of the cations at 0.1 M is also unknown but likely to be less than unity. It is also not at all certain that the concentration of ions in the internal water space is given by that fraction of the total uptake which requires metabolic uptake.

The potentials calculated by insertion of the cation concentrations into the Nernst equation (neglecting activity coefficients) do not agree very closely: DDA^+ distribution, for instance in Fig. 3, corresponds to a potential of -155 mV whereas accumulation of K^+ in the presence of

valinomycin (Table 2) indicates a potential in the neighborhood of -200 mV. On balance we believe that an attempt to resolve the discrepancy would be premature. The fact that valinomycin catalyzes very rapid equilibration (half time less than 1 min) whereas DDA^+ equilibrates rather more slowly favors the higher potential. But it is not certain whether the very active system which transports K^+ into the cells can be totally disregarded: the complexities of this system will be considered in the following paper. Besides, there is evidence that, at least in *Halobacteria*, part of the cellular K^+ may be bound to cytoplasmic constituents (Cope & Damadian, 1970; Ginzburg, Sachs & Ginzburg, 1971). Whatever may be the correct value, both the K^+ and the DDA^+ potentials are far higher than those calculated a decade ago from the distribution of chloride in *E. coli* (Schultz, Wilson & Epstein, 1962).

Finally, a comment is in order concerning the displacement of DDA^+ from the cells by K^+ (Fig. 3) and by lowering the external pH (Fig. 7). In the following paper (Harold & Papineau, 1972) we shall present reasons for believing that extrusion of protons from the cells is the basic process responsible for generation of the potential difference. It should be recalled that during glycolysis, large amounts of H^+ are generated. Presumably at pH 6.0, either extrusion of H^+ is inhibited or else the rate of backflow of H^+ is sufficient to keep the cells from maintaining the potential difference. Similarly, the observation that cells replete with K^+ do not accumulate DDA^+ suggests that K^+ uptake occurs in such a manner as to collapse the electrical potential: only when valinomycin is present does DDA^+ freely displace K^+.

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