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Induction of Solute Release from *Nicotiana tabacum* Tissue Cell Suspensions by Polymyxin and EDTA

RALPH WEIMBERG[1]

U.S. Salinity Laboratory, 4500 Glenwood Drive, Riverside, CA 92501, U.S.A.

H. R. LERNER AND A. POLJAKOFF-MAYBER

Botany Department, Hebrew University of Jerusalem, Jerusalem, Israel

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ABSTRACT

For determination of the effects of polymyxin B, polymyxin E, or ethylenediamine tetra-acetic acid (EDTA) on plant cell membranes, the rates at which three solutes, K^+, P_i, and sugar, leaked from treated tissue culture cell suspensions of *Nicotiana tabacum* were measured. The kinetics of leakage from cells treated with either of the polymyxins was biphasic, whereas kinetics for cells treated with EDTA was monophasic. Only K^+ leaked from polymyxin-treated cells during the first phase, and all three solutes leaked during the second phase. The slower first phase is interpreted as leakage of K^+ from the Donnan free space and cytoplasm, and the faster second phase as the leakage of solutes from the vacuole. The monophasic kinetics of EDTA treatment indicated that solutes were leaking simultaneously from cytoplasm and vacuole. Of the divalent cations tested, only Ca^{++} and Mn^{++} counteracted the effects of polymyxin and EDTA. Ca^{++} even restored P_i and sugar uptake. Addition of Mg^{++} or Sr^{++} to polymyxin-treated cells did not stop solute leakage but actually enhanced the leakage rates. A model is presented that suggests that polymyxin or EDTA induces solute leakage by forming pores in plant cell membranes. The effects of divalent cations on membranes once the pores are formed are also discussed.

Key words: Polymyxin; EDTA; *Nicotiana tabacum*; Solute leakage.

INTRODUCTION

We have been studying pore formation in plant cell membranes as induced by poly-L-lysine, amphotericin B, (Lerner and Reuveni, 1982; Lerner, Ballarin-Denti, and Pradet, 1983) and toluene (Lerner, Ben-Bassat, Reinhold, and Poljakoff-Mayber, 1978; Weimberg, Lerner, and Poljakoff-Mayber, 1981, 1982). In a continuing investigation to screen for other compounds that perturb membranes, we have now initiated studies on pore formation in cell membranes of cell tissue suspension of *Nicotiana tabacum* by polymyxins B and E and by ethylenediamine tetra-acetic acid (EDTA). We have become interested in these compounds because of their ability to induce pores in bacterial and artificial membranes.

The polymyxins are polycationic, polypeptide antibiotics (see Storm, Rosenthal, and Swanson, 1977, for a discussion of their chemistry and properties). It is believed that their bacteriocidal action and their ability to perturb cell membranes resulting in pore formation are related. A model, based on studies on the interaction of polymyxin with lipid monolayers

[1] To whom reprint requests should be sent.

(Treuber and Miller, 1977) and bilayers (Imai, Inoue, and Nojima, 1975; Hartman, Galla, and Sackman, 1978; Sixl and Galla, 1979; Galla and Trudell, 1980), has been proposed to explain their perturbation and, thus, bacteriocidal action. According to the model, the binding of polymyxin molecules to the membrane is due to two types of interactions: (a) hydrophobic interactions of the fatty acid moiety of the polymyxin molecules with the fatty acid chains of the lipids; and (b) the electrostatic interactions of the cationic sites of the antibiotic with the anionic sites of acid phospholipids. Clustering of polymyxin molecules with negatively charged lipids causes phase separation. Moreover, the asymmetry of the polymyxin molecule imposes a distortion in the lipid matrix of the bilayer that results in pore formation in liposomes and biological membranes. In nature divalent cations (Ca^{++}, Mg^{++}) are usually bound to the membrane anionic sites; this prevents charge repulsions and therefore maintains membrane tightness. As polymyxin binds to a membrane, it displaces the inorganic divalent cations. Conversely, addition of divalent cations to perturbed membranes displaces bound polymyxin molecules and thus counteracts the effect of pore formation.

Another compound that also significantly alters permeability properties of bacterial membranes is EDTA (Leive, 1974). The suggested mechanism for EDTA is that it removes membrane-bound Ca^{++} by chelation. Because Ca^{++} is needed for membrane integrity, its removal from the membrane alters the membrane so that fairly large molecules can now easily penetrate and enter the cell where previously they could not. The effects of polymyxin and EDTA on bacterial cells are so similar that they can be used interchangeably (Leive, 1974; Storm *et al.*, 1977). For instance, periplasmic enzymes can be eluted from *Escherichia coli* by either polymyxin B (Cerney and Teuber, 1971) or by EDTA plus osmotic shock (Leive, 1974). The effects of either compound on bacteria can be counteracted with Ca^{++} and other divalent cations. They differ, mainly, in that EDTA, if used correctly, is not lethal to bacteria.

Because of these previous reports on their effects on bacterial cells, we decided to see if polymyxin and EDTA were capable of also altering plant cell membranes. We found that polymyxin and EDTA do perturb membranes of plant tissue cells in suspension, allowing the efflux of solutes. This communication will describe the kinetics of solute leakage due to pore formation by polymyxins and EDTA as well as the counteraction by divalent cations on these systems.

MATERIALS AND METHODS

Nicotiana tabacum, cv. Xanthi, tissue culture suspensions were grown aerobically in Gamborg's medium B-5 (Gamborg, Miller, and Ojima, 1968) at 25 °C. They were harvested after 8–13 d incubation (in the stationary phase of growth) by gravity filtration on Miracloth.[1] The cells were washed thoroughly with water while on the filter cloth, suspended in water, and centrifuged at 2000 rev. min^{-1} (600 × *g*) for 5 min in order to measure packed volume. The packed cells were suspended in H_2O at a concentration of 30 ml packed cell volume per 100 ml suspension.

For time-course experiments, 250 ml Erlenmeyer flasks containing 40 ml of cell suspension each were gently shaken at 30 °C on a reciprocal shaker to maintain aerobic conditions. Small volumes of concentrated solutions of the various reagents were added so that the final volumes were not increased by more than 1%. The flasks were covered with parafilm to prevent evaporation. Aliquots of 4·0 ml were removed at intervals and filtered by gravity through Miracloth to harvest cells. The filtrates were used for solute assays. To measure the total amounts of solutes in cells, aliquots of untreated cell suspensions were heated in a boiling water bath for 10 min, cooled, and filtered through Miracloth; the filtrates used for the assays.

Polymyxin B (PM-B) and polymyxin E (PM-E) were commercial preparations obtained from Sigma Chemical Corporation[1]. PM-B was purchased as polymyxin B sulphate, 7700 units per mg, and PM-E

[1] Company names or products are used for the benefit of the reader and such uses do not imply endorsement, guarantee or preferential treatment by the United States Department of Agriculture or its agent.

was purchased as colistin methane sulphonate, sodium salt, 11 000 units per mg. Potassium was assayed by atomic absorption spectroscopy. Standard chemical methods were used for the determination of P_i (Taussky and Shorr, 1953), sugars (Hassid and Abraham, 1957) and amino acids (Spies, 1957). Phosphate measurements in filtrates from PM-E treated cells were of only semi-quantitative value because these filtrates contained a compound that produced turbidity with the phosphate-detecting reagents.

All experiments were replicated. The data are not presented statistically because the amounts of solutes present in cells and the rates at which they leaked varied with batches of cells. The variation in K^+ in hot water extracts of different batches of cells ranged from 45–92 μmol ml^{-1} packed cells, and the variations in P_i and sugars ranged from 2·0–3·4 and 3·5–13 μmol ml^{-1} packed cells respectively. Therefore, the results of individual experiments only are reported. Despite these variations in quantitative data, the kinetics of solute leakage in time-course experiments were reproducible.

RESULTS

Titration of the amount of PM-B and EDTA needed to induce solute leakage

Washed *Nicotiana* cells were incubated with various amounts of either PM-B or EDTA, and the rates of leakage from the cells and the total amounts present in the cells of K^+, P_i, and total sugars were determined. The patterns of leakage of solutes from cells treated with PM-B depended on the concentration of the antibiotic. With concentrations of PM-B of 100 μg ml^{-1} packed cells or more, the three solutes began leaking out essentially as soon as the antibiotic was added to the reaction mixture (Fig. 1). About 4 h of incubation was required for maximal amounts of these solutes to leak from the cells. Essentially all cellular K^+ and P_i could be recovered in reaction mixture supernatants. The maximal amounts of sugars to leak from treated cells was 50–70% of the total as determined by hot water extraction of untreated cells. Increasing the amount of PM-B added initially to cells to 400 μg ml^{-1} increased the rate of leakage but had no effect on total amounts of solutes leaked.

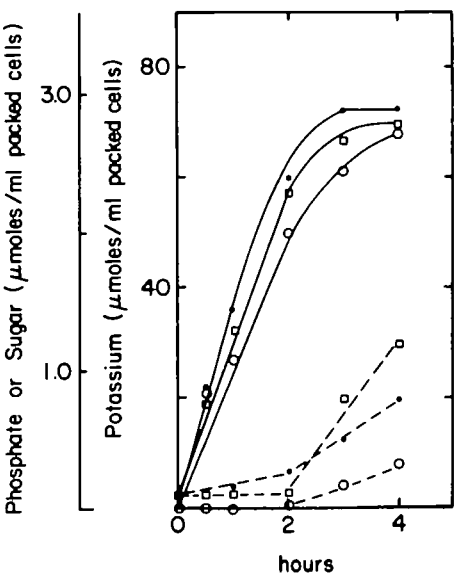

FIG. 1. Induction of leakage of solutes by PM-B. PM-B was added to cells at zero time. Solid lines, 200 μg ml^{-1} packed cells of PM-B; dashed lines, 60 μg ml^{-1} packed cells of PM-B. Closed circles, K^+ leakage; open circles, P_i leakage; and squares, sugars. Total amounts of solutes in cells as determined by a hot water extraction: K^+, 78 μmol ml^{-1} packed cells; P_i, 2·9 μmol ml^{-1}; and sugars, 4·3 μmol ml^{-1}.

At concentrations of PM-B below 100 μg ml^{-1} packed cells, the effect of the antibiotic was biphasic; i.e. there were two distinguishable rates of solute leakage (Fig. 1, 60 μg ml^{-1} of PM-B). The initial slow rate lasted until 10–15% of the total cellular K^+ had leaked out and then the second, fast rate began. As the amount of PM-B in the reaction mixture was lowered, the first rate also decreased and, consequently, the length of time for the leakage of 10–15% of cellular K^+ became longer. At 30 μg ml^{-1} PM-B, the rate during the first phase was so slow that 10% of cellular K^+ had not yet leaked out after 4 h incubation, the time at which the experiment of Fig. 1 was terminated (data not shown). The beginning of P_i leakage usually coincided with the beginning of the second rate of K^+ leakage. The pattern of sugar leakage was similar to that of P_i, though its correlation with the beginning of the second phase was not as clear-cut. There was always a low amount of leakage of sugars during the first phase, but the correlation of the beginning of sugar leakage with the beginning of P_i leakage grew closer as the amount of PM-B being used to treat the cells became less and less.

Though only a few points were available, plotting the reciprocal of the rate of leakage versus the reciprocal of the PM-B concentration yielded a straight line (Fig. 2) from which an approximate apparent K_m value for the system could be extrapolated. This apparent K_m value was about 1000 μg ml^{-1} PM-B for K^+ leakage during the first phase as well as for K^+, P_i, and sugar leakage during the second phase.

Na-EDTA, pH 7·0, induced leakage from *Nicotiana* cells when used at a concentration of 0·25 mM or more (Fig. 3). The rates of leakage of all three solutes increased with increasing

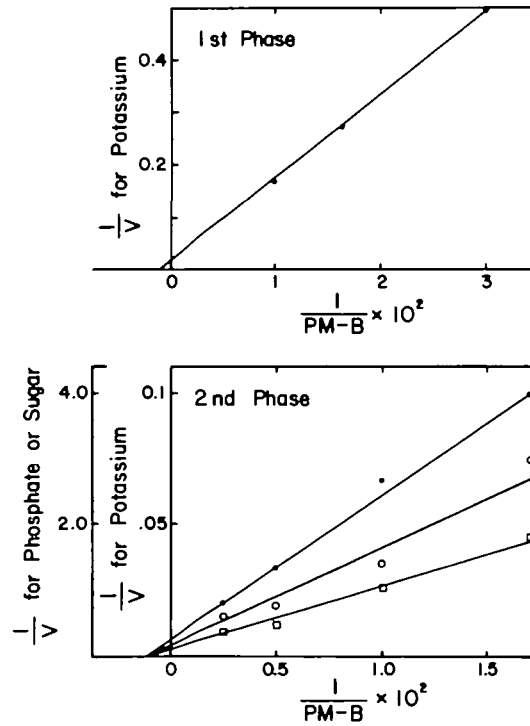

FIG. 2. Rates of solute leakage during first and second phases of the reaction as a function of PM-B concentrations. Concentrations of PM-B are μg ml^{-1} packed cells; rates are μmoles ml^{-1} packed cells h^{-1}. Closed circles, K^+ leakage; open circles, P_i; squares, sugars.

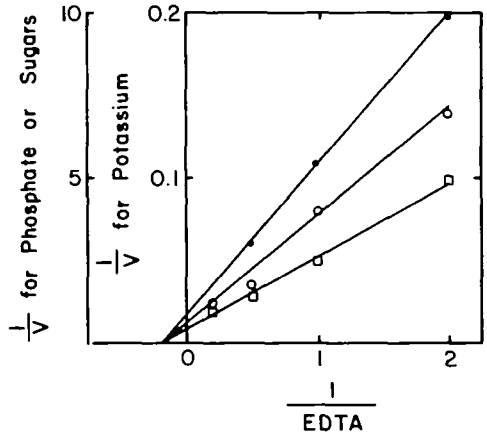

FIG. 3. Rates of solute leakage as a function of EDTA concentrations. Concentrations of Na-EDTA (pH 7·0) are mM; rates are μmoles ml^{-1} packed cells h^{-1}. Symbols same as Fig. 2.

Na-EDTA concentrations, but, unlike the biphasic effects of PM-B, leakage of solutes due to EDTA treatment began immediately upon addition of EDTA to the reaction mixtures and continued at constant rates until almost all of the extractable amount of solute had been released from the cells. The total amount of each solute leaked was similar to that leaked by the PM-B treatment. The rate of amino acid leakage induced by Na-EDTA was also measured and it followed the same patterns as the sugars (not shown). Reciprocal rate versus concentration plots were linear and all three were extrapolated to the same apparent K_m value of about 10 mM Na-EDTA (Fig. 3).

Effect of Ca^{++}

$CaCl_2$ completely protected *Nicotiana* cells from the effects of PM-B if added to reaction mixtures in high enough concentrations. As the concentrations of $CaCl_2$ were lowered, the degree of protection decreased and the pattern of solute leakage became biphasic regardless of the concentration of PM-B. Plots of $1/v$ versus 1/PM-B at various Ca^{++} concentrations seemed to indicate that the mechanism of Ca^{++} protection was the competition by the inorganic cation for the PM-B binding sites (Fig. 4; only the data for K^+ leakage during the second phase are presented). The apparent K_i of Ca^{++} as an inhibitor of PM-B action was approximately 10 mM (Fig. 5).

$CaCl_2$ also antagonized the induction of leakage by EDTA, but the kinetics were different from those for the effect of $CaCl_2$ on cells treated with PM-B. A concentration of 10 mM $CaCl_2$ completely blocked the effect of 5·0 mM EDTA. The patterns of leakage were monophasic at all concentrations of $CaCl_2$. Between 0·0 mM and 7·0 mM $CaCl_2$ the protective effect of $CaCl_2$ increased proportionately with its concentration (Fig. 6).

When added to PM-B or EDTA treated cells 1 h after the beginning of the treatment, 20 mM $CaCl_2$ gradually slowed down and finally stopped the further leakage of P_i and sugars from the cells (Fig. 7). Accompanying this, there was actually a change in net flow of these two solutes from efflux to influx. Within 1 h of adding $CaCl_2$ to EDTA-treated cells, the cells had re-absorbed almost all the P_i that had previously leaked from the cells along with a major proportion of the sugars. The effect of $CaCl_2$ was the same on PM-B-treated cells except that re-absorption occurred more slowly and was not as efficient.

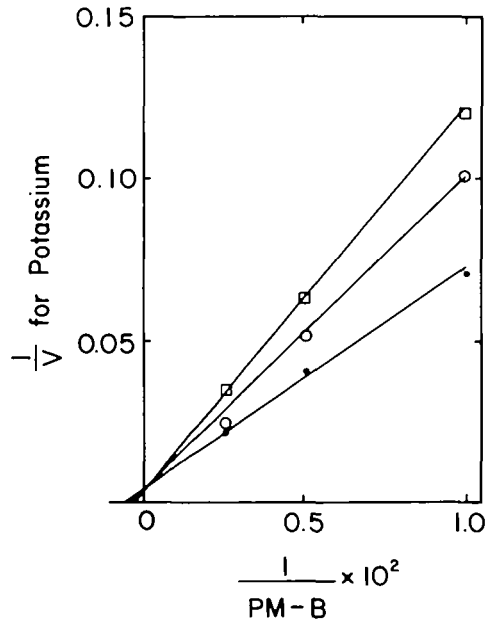

FIG. 4. Effect of $CaCl_2$ on K^+ leakage from PM-B treated cells. PM-B and $CaCl_2$ were added to reaction mixtures together at zero time. Rates were measured during the second phase of solute leakage. Concentrations of PM-B are μg ml^{-1} packed cells; rates are μmoles ml^{-1} packed cells h^{-1}. Closed circles, no $CaCl_2$; open circles, 5·0 mM $CaCl_2$; squares, 10 mM $CaCl_2$.

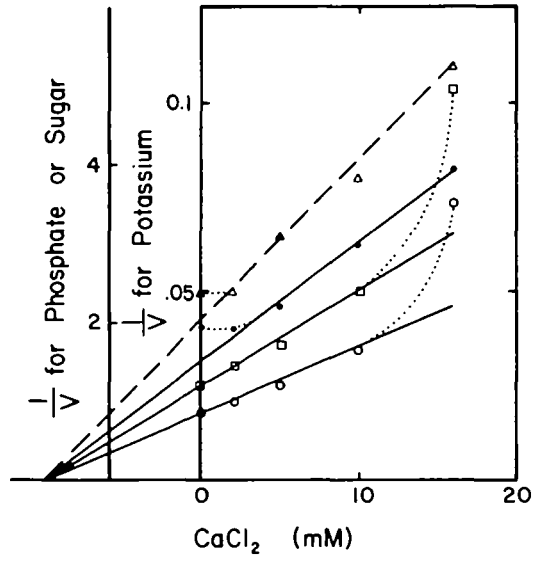

FIG. 5. Inhibition of PM-B action by $CaCl_2$. PM-B and $CaCl_2$ were added to reaction mixtures simultaneously. Rates were measured during second phase of solute leakage and are measured in μmoles ml^{-1} packed cells h^{-1}. Solid lines, 200 μg ml^{-1} PM-B, dashed line, 400 μg ml^{-1} PM-B. Closed circles and triangles, K^+; open circles, P_i; squares, sugar.

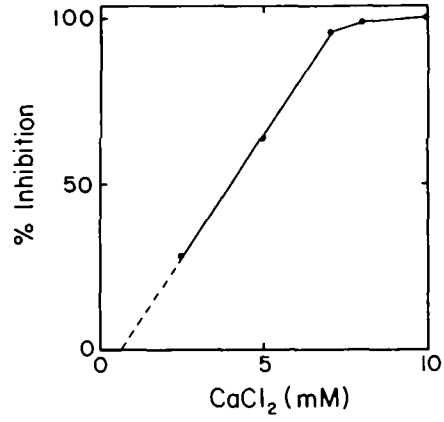

FIG. 6. Inhibition by $CaCl_2$ of K^+ leakage induced by EDTA. Na-EDTA (pH 7·0) and $CaCl_2$ were added to reaction mixtures simultaneously at zero time. Concentration of EDTA is 5·0 mM. Rates are μmoles ml^{-1} packed cells h^{-1}.

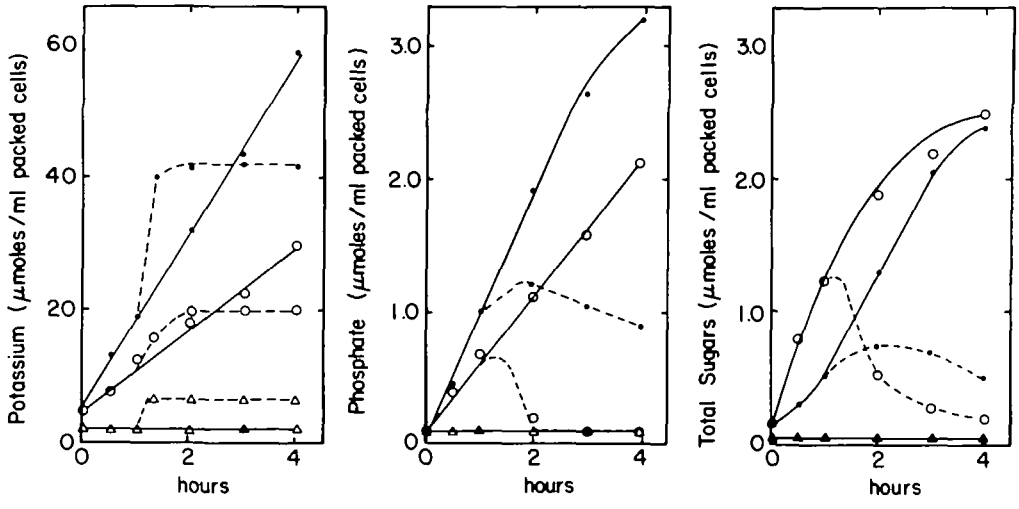

FIG. 7. Effect of $CaCl_2$ on the leakage of solutes from PM-B or EDTA treated cells when added to reaction mixtures 60 min after perturbing agent. Solid lines, leakage of solutes in absence of $CaCl_2$; dashed lines, leakage in presence of $CaCl_2$. Concentration of PM-B, 200 μg ml^{-1}; EDTA, 5·0 mM; $CaCl_2$, 20 mM. Closed circles, PM-B treated cells; open circles, EDTA-treated cells; triangles, control cells (cells not exposed to either perturbing agent).

K^+ leakage from perturbed cells was also stopped by $CaCl_2$ but in a different manner than that for P_i or sugars. Addition of $CaCl_2$ to reaction mixtures 1 h after the perturbing agent caused an abrupt increase in the rate of K^+ leakage, which lasted for 20–60 min and then there was a relatively sudden stop to any further increase in K^+ levels in the medium. Unlike with P_i and sugars, there was no net re-absorption of K^+ back into the cells. The level of K^+ in the medium remained constant. The quantity of K^+ released from cells as a result of adding $CaCl_2$ to reaction mixtures was a function of the amount of K^+ leaked before $CaCl_2$ was

added. The minimal amount that was released in this manner was the amount that can be eluted from untreated cells which was about 5% of total cellular K^+.

Effect of other divalent cations

In bacteria other divalent cations besides Ca^{++} could counteract the bacteriocidal effect of polymyxin (HsuChen and Feingold, 1972; Newton, 1954, 1956; Davis, Ianetta, and Wedgwood, 1971). Therefore, the ability of Mn^{++}, Mg^{++}, and Sr^{++} to protect *Nicotiana* cells from the perturbing effect of PM-B was studied (Fig. 8). Mn^{++} at 20 mM was more effective

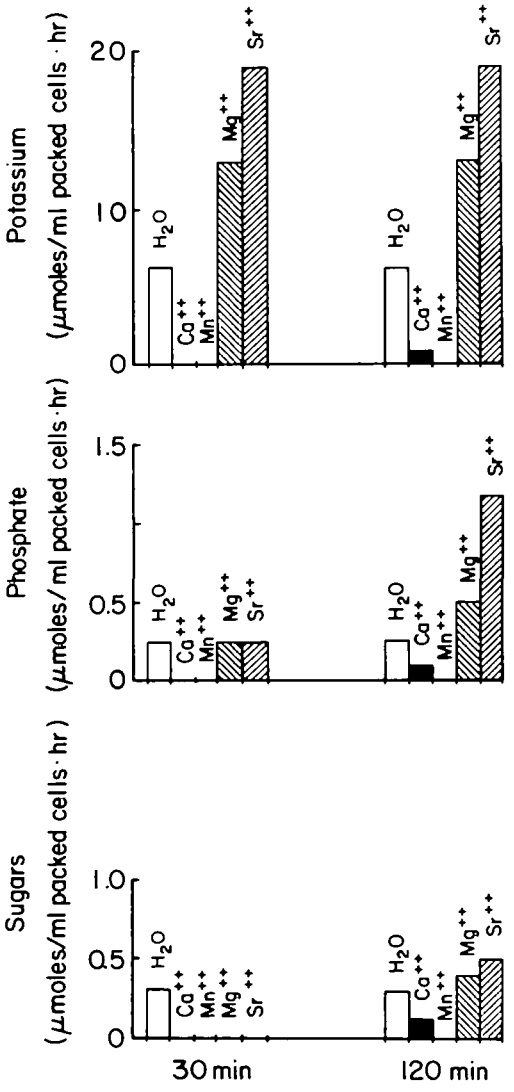

FIG. 8. Rates of leakage of solutes treated with PM-B in the presence or absence of divalent cations. Polymyxin and cation added simultaneously at zero time. The first set of histograms are leakage rates after 30 min incubation, and the second set are the leakage rates after 120 min incubation. Concentration of PM-B, 100 μg ml^{-1}; concentration of divalent salts, 20 mM. The divalent cations were used as their chloride salts.

than Ca^{++} in preventing leakage of solutes. Mg^{++} and Sr^{++}, on the other hand, stimulated solute leakage. In the presence of Mg^{++}, K^+ leaked from PM-B-treated cells at a rate that was twice that of control cells; in the presence of Sr^{++} the rate was 3 times higher. The patterns of K^+ leakage from PM-B-treated cells in the presence of Mg^{++} or Sr^{++} were monophasic but biphasic in the presence of Ca^{++}. On the other hand, the patterns of leakage of P_i and sugars from perturbed cells in the presence of Mg^{++} or Sr^{++} were different from each other and from K^+ leakage. For the first 60 min after addition of PM-B plus either $MgCl_2$ or $SrCl_2$ to cells, the rates of leakage of P_i were the same as from cells treated with PM-B alone. Gradually over an additional period of 1 h, the rates accelerated until they were 2 fold greater in cells being treated with PM-B in the presence of Mg^{++}, and 5 fold greater in those being treated with PM-B plus Sr^{++}. The rate remained constant in the cells being treated with PM-B alone.

There was no leakage of sugars from cells for the first 90 min of incubation with PM-B if any one of the four divalent cations was also added to reaction mixtures simultaneously with the antibiotic. Between 90 min and 120 min of incubation, the rates of sugar leakage from cells in reaction mixtures containing either Mg^{++} or Sr^{++} accelerated to values somewhat higher than that from cells in a reaction mixture without divalent cation.

The above results were obtained when the concentration of divalent cation in reaction mixtures was 20 mM and the cation was added to reaction mixtures simultaneously with PM-B. At this concentration, the effects were maximal. The described effects decreased with lower concentrations until, at 2·0 mM, there was no measurable effect by any divalent cation on the induction of solute leakage by PM-B.

When these divalent cations at a concentration of 20 mM were added to PM-B treated cells 1 h after the polymyxin, their effects fell into two patterns (Fig. 9); however, all four cations caused the abrupt increase in the rate of K^+ leakage from both untreated and treated cells. With Ca^{++} and Mn^{++}, further K^+, P_i, and sugar leakage from cells stopped within 60 min after adding the cations. There was, in addition, a re-absorption of P_i and sugar, but only in cells in reaction mixtures to which Ca^{++} had been added. For Mg^{++} and Sr^{++}, K^+, P_i, and sugar leakage rates increased abruptly upon adding these cations to PM-B-treated cells.

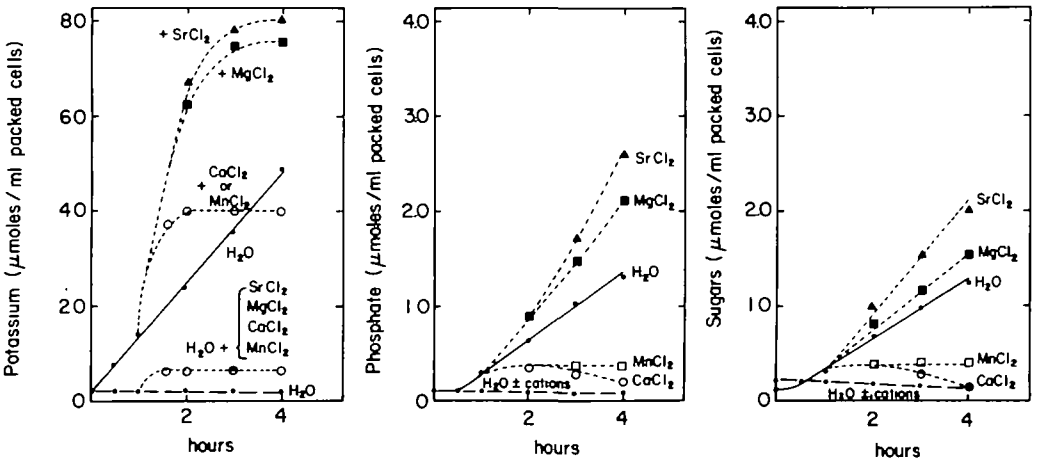

FIG. 9. Effect of various divalent cations on solute leakage induced by PM-B when added to reaction mixtures 1 h after the antibiotic. Concentration of PM-B, 100 μg ml^{-1} packed cells; concentration of salts, 20 mM. Solid line, leakage from cells treated with PM-B only; dashed line, leakage from control cells (cells not exposed to PM-B); dotted lines, leakage from cells after adding divalent cation to reaction mixtures. There was no leakage of P_i or sugar from control cells treated with divalent cations.

Solute leakage from PM-E-treated cells

Qualitatively, induction of leakage of solutes from *Nicotiana* by PM-E was similar to that by PM-B. However, there were major quantitative differences. It required almost 10 times more PM-E than PM-B (on a weight basis) to induce leakage from cells. The patterns of leakage of the three solutes measured were biphasic even at the highest concentration of PM-E used (3·0 mg ml^{-1} packed cells). As with PM-B, the second phase did not begin until 10–15% of total cellular K^+ had leaked from the cells. The apparent K_m of the system for PM-E was 10 mg ml^{-1}; this also was 10 fold greater than the apparent K_m for PM-B. Only K^+ and sugars could be measured in supernatants of reaction mixtures. P_i could not be measured in reaction mixtures containing more than 1·0 mg ml^{-1} PM-E because other unidentified constituents were present in reaction mixtures that formed a precipitate with the reagents for P_i determination. However, as well as it could be determined by visual observation, P_i leaked from PM-E-treated cells in a biphasic pattern similar to that described for PM-B cells.

Ca^{++} and Mn^{++} protected cells from the pore-inducing action of PM-E but the kinetics were different from those obtained with PM-B-treated cells. A concentration of 2·0 mM $CaCl_2$ or $MnCl_2$ was adequate to provide 100% protection to PM-E-treated cells (Fig. 10, only the effect of $CaCl_2$ on K^+ leakage is shown). The effect of Ca^{++} at concentrations lower than 2·0 mM seemed to be limited to the first phase of leakage. The higher the concentration of Ca^{++}, the longer was the first phase. Once the second phase began, though, the rates of leakage were essentially the same at all concentrations of $CaCl_2$. If the apparent K_i of $CaCl_2$ was 10 mM for PM-E during the second phase as it was for PM-B, these concentrations of $CaCl_2$ would be too low to have any measurable effect on leakage rates. Mg^{++} and Sr^{++} at a concentration of 2·0 mM had neither stimulatory nor protective effects on rates of solute leakage. It required concentrations of 20 mM $MgCl_2$ or $SrCl_2$ to stimulate solute leakage from

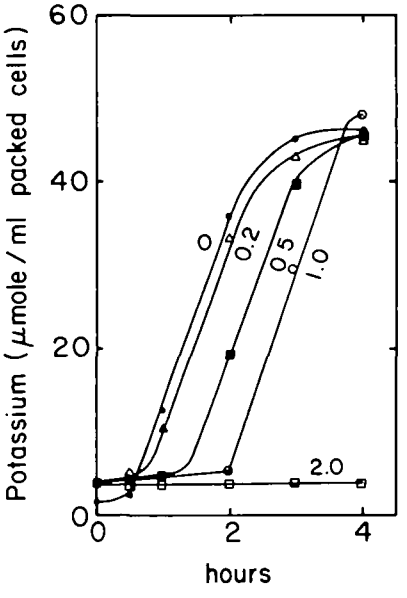

FIG. 10. Effect of $CaCl_2$ on K^+ leakage from cells treated with PM-E. PM-E and $CaCl_2$ were added to cells simultaneously. Concentration of PM-E, 3·0 mg ml^{-1} packed cells. The number on each curve is the concentration of $CaCl_2$ in mM.

PM-E treated cells, and the patterns observed were similar to those shown in Figs 8 and 9 for PM-B treated cells.

DISCUSSION

The effects of polymyxin on *Nicotiana* tissue culture cells in suspension is the same as those on bacterial cells in the following respects: (1) Low molecular weight compounds leaked out of treated cells; (2) The rates of leakage increased with increasing polymyxin concentrations; and (3) The effect of polymyxin could be counteracted by Ca^{++}. One significant difference was that the kinetics of solute leakage when low amounts of PM-B or any concentration of PM-E were used were biphasic in plant cells and monophasic in bacteria. In *Nicotiana* about 10% of total cellular K^+ and a small amount of sugar leaked from cells in the first phase. This amount of K^+ roughly corresponds to the cytosolic K^+ pool (Lerner and Reuveni, 1982) or, as will be explained later, actually the cytosolic pool plus K^+ in the Donnan free space. The first phase always lasted until this amount of K^+ had been released no matter how slow the first rate was. The beginning of the second phase could be recognized by an increase in the rates of K^+ and sugar leakage plus the appearance of P_i in the reaction mixture.

As we interpret these data, the first phase represents solute leakage from the cytoplasm, and the second phase is the release of solutes from the vacuole. Vacuolar leakage does not begin until the first phase has ended, and the beginning of the second phase is controlled by the decrease in the concentration of cation in the cytoplasm. We propose the following model as an explanation of these observations and conclusions.

When *Nicotiana* cells are exposed to low concentrations of polymyxins, the antibiotic binds to the outer side of the plasmalemma and only small pores are formed. On the inner side of the plasmalemma, the cytosolic cations compete with polymyxin and prevent the antibiotic from binding to sensitive sites (Treuber and Miller, 1977). The cytosolic K^+ pool concentration in these cells is initially about 190 mM and the vacuolar K^+ pool concentration is about 110 mM (Lerner and Reuveni, 1982). Leakage is relatively slow because the plasmalemma is rich in sterols (Hodges, Leonard, Bracker, and Keenan, 1972) and the presence of sterols causes a lowering of the rates of solute leakage (Imai *et al.*, 1975). The passage of solute molecules through the plasmalemma is, then, the rate-limiting step during the first phase. When most of the cytosolic cations have leaked out and their pools have reached concentrations low enough that they no longer can compete with the antibiotic, polymyxin molecules begin to bind to both inner and outer sides of the plasmalemma as well as to the tonoplast. The binding of polymyxin to both faces of the plasmalemma permits the rate of leakage through this membrane to increase so that it no longer is the rate-limiting step. At the same time, vacuolar solutes begin to leak through the tonoplast. Since the tonoplast contains much lower amounts of sterols than the plasmalemma, polymyxin-induced leakage through the tonoplast is faster than it had been through the plasmalemma.

When high concentrations of PM-B were used (200 $\mu g\ ml^{-1}$), the slow initial phase of K^+ leakage was not observed. This may be due to an acceleration of the rate of the entire first phase to the point that it is too short to be detected under the sampling conditions of these experiments.

Eventually, all cellular K^+ and P_i are released by polymyxin treatment. From the biphasic kinetics, it would appear that 90% of K^+ and all P_i are in the vacuole. There may be some P_i in the cytoplasm since Lerner and Reuveni (1982) claim that 2% of cellular P_i is in this compartment. However, the experimental error in these experiments is too large to permit reliable measurements of such a proportion. About 63% of total anthrone-positive compounds leaked from treated cells, mostly from the vacuolar compartment. It may be

assumed that the other 37% are water-soluble polysaccharides extractable with hot water but too large to diffuse through pores in membranes generated with PM-B.

The difference between the action of PM-B and PM-E on *Nicotiana* cells is mainly in the amount of antibiotic needed to induce leakage of K^+ during the initial phase. Ten times more PM-E than PM-B (on a weight basis) was required to induce leakage. PM-E, as purchased commercially, is a methane sulphonate derivative of the antibiotic with all cationic groups covered. However, there is a slow hydrolysis of the derivative back to its polycationic form in aqueous solution (Barnett, Bushby, and Wilkinson, 1964). We assume that it is only the free PM-E in solution that is active on *Nicotiana* cells, and that the kinetics observed are due to its very low concentration in reaction mixtures.

Unlike the kinetics of leakage from PM-B treated cells, the kinetics of solute leakage from EDTA-treated cells was monophasic. The monophasic pattern of leakage indicated that EDTA produced pores in the plasmalemma and tonoplast essentially simultaneously. This would be possible if EDTA produced pores very rapidly and the size of the pores in the plasmalemma were large enough to provide no hindrance to the diffusion of EDTA into the cells.

Ca^{++} protected tissue culture cells from the induction of leakage by both polymyxin and EDTA. It probably inhibited the EDTA effect by chelating with the EDTA and, thus, effectively reducing the amount of EDTA left in solution. However, the mechanism of protection of polymyxin-treated cells by Ca^{++} is different because there is no interaction between PM-B and Ca^{++} (Sixl and Galla, 1981). The kinetics of the protective reaction indicated that there is a competition between PM-B and Ca^{++} for sensitive sites on the cell membrane and these sensitive sites, according to Hartmann *et al.* (1978) are the acid groups of membrane phospholipids. Pore formation is due to a distortion of the lipid matrix of membranes when polymyxin is attached to these sites. In the absence of added Ca^{++} in the reaction mixture, the ratio of PM-B to cellular Ca^{++} would be high enough to permit replacement of the membrane Ca^{++} with PM-B. The inclusion of Ca^{++} in reaction mixtures would protect cells by shifting the equilibrium of attachment to the sites in favour of Ca^{++}.

EDTA is probably reacting with membranes at the same acid phospholipid sites but producing pores by a different mechanism. EDTA undoubtedly is removing Ca^{++} from the sensitive sites by chelation which leaves the membrane with multiple negative charges. This leads to charge repulsion resulting in pore formation and leaky membranes. Further evidence that PM-B and EDTA are reacting with the same sites was obtained by adding PM-B and EDTA to cells simultaneously. Under these conditions, the patterns of solute leakage were monophasic, and the effect on rates of leakage was synergistic (unpublished results).

The perturbation of membranes caused by PM-B or EDTA is reversible. Addition of Ca^{++} to leaking cells 1 h after the perturbing agent resulted not only in a blocking of further solute leakage, but, actually, in a reabsorption of two of the solutes, P_i and sugars. It was as if Ca^{++} had 'repaired' the membranes. Ca^{++} was undoubtedly binding to uncovered phospholipid sites in EDTA-treated cells or displacing the antibiotic from phospholipid in PM-B treated cells. This relieved the perturbation allowing the membrane to recover some of its normal functions. Recovery of absorption abilities for P_i and sugars could be achieved by adding Ca^{++} to reaction mixtures even as long as 8 h after the perturbing agent—long after all of the three solutes had leaked from the cells (unpublished results). Why the cells never recovered their ability to absorb K^+ is not known.

Ca^{++} had another effect on membranes besides inhibiting the induction of leakiness due to polymyxin or EDTA. Adding Ca^{++} to untreated cells or to cells simultaneously with the perturbing agents resulted in an abrupt release of 5% of the total cellular K^+. Because this K^+

is released before pores were generated in membranes, it is reasonable to assume that this K^+ is from the cell wall and cell surface, the so-called Donnan free space (Winter, 1961). If Ca^{++} is added to cells 1 h after the perturbing agents, then a greater amount of K^+ was rapidly released. The source of the larger amounts of K^+ from treated cells is probably internal K^+ that became attached to negative sites in the Donnan free space as it diffused outward through pores. If K^+ is bound to the Donnan free space, and that process is part of the pathway of K^+ movement from the cell interior to the exterior solution, then the rates of appearance of K^+ in the medium may not be a true measure of the rates of K^+ leakage through the pores.

Of the four divalent cations tested, only Ca^{++} and Mn^{++} protected *Nicotiana* cells from the perturbing actions of polymyxin and EDTA. Not only did Mg^{++} and Sr^{++} not protect the cells from the perturbing agents, they actually enhanced the rates of solute leakage. In bacteria, all four cations counteracted the action of PM-B even though they were not equally effective (Newton, 1954). It is significant that *Nicotiana* cells had to be perturbed first in order to observe this effect by Mg^{++} or Sr^{++}. It is obvious from the experimental results that the correct conformation of the membrane to prevent leakiness is achieved with Ca^{++} and Mn^{++} but not with Sr^{++} or Mg^{++}. However, only in the presence of Ca^{++} was the membrane repaired sufficiently to allow solute reabsorption.

All four cations do bind to the cell surface for they all caused the abrupt release of K^+ from the Donnan free space in exactly the same manner and to the same extent. Thus, in perturbed cells, K^+ appeared to leak faster from cells when Mg^{++} or Sr^{++} were present than when they were absent because the attachment sites for K^+ in the Donnan free space were occupied by these cations.

From this study it appears that the use of low concentrations of polymyxin can permit the differention between cellular compartments. Similar results were obtained with poly-L-lysine (Lerner and Reuveni, 1982) except that the effect of this pore-inducing polymer was restricted to the plasmalemma. A more detailed study of the interaction and competition between polymyxin and the divalent cations on plant cell membranes may reveal information concerning the organization of plant cell membranes. It appears, therefore, that polymyxin may prove to be just as useful a technique for studying plant cell membranes as it has been for bacterial and artificial membranes.

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