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On the Mode of Action of the Bacteriocin Butyricin 7423

Effects on Membrane Potential and Potassium-Ion Accumulation in *Clostridium pasteurianum*

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1. The apparent transmembrane bulk-phase electrical potential ($\Delta\psi$) of *Clostridium pasteurianum* was determined from the distribution ratio of the membrane-permeable cation butyltriphenylphosphonium ($BuPh_3P^+$). In glycolysing cells the highest value of $\Delta\psi$, calculated on the assumption that there was no energy-dependent binding of $BuPh_3P^+$ to the organisms, was recorded in media containing only 2–3 mM K^+ ions and, even so, was only 100–110 mV.

2. Efrapeptin, a BF_1-directed inhibitor of the membrane H^+-ATPase of *Cl. pasteurianum*, abolished the membrane potential ($\Delta\psi$) and caused complete efflux of actively-transported K^+ ions. Thus protonmotive hydrolysis of ATP generated by substrate level phosphorylation is the sole means of membrane energisation in this anaerobe.

3. At low (sublethal) concentrations, butyricin 7423 stimulated K^+ efflux from *Cl. pasteurianum* without measurably affecting its membrane potential. At lethal and supralethal concentrations of this bacteriocin, both $\Delta\psi$ and active K^+ uptake were abolished.

4. Whilst the addition of valinomycin to cells of *Cl. pasteurianum* suspended in media of low K^+ concentration generated a diffusion potential to which $BuPh_3P^+$ would respond, addition of butyricin 7423 in place of valinomycin caused no such effect. Also, unlike valinomycin, butyricin 7423 did not increase the rate of K^+ efflux from non-glycolysing cells of *Cl. pasteurianum*. Valinomycin stimulated, but butyricin 7423 inhibited, the uptake of $^{86}Rb^+$ ions by glycolysing cells of *Cl. pasteurianum*.

5. A mutant strain of *Cl. pasteurianum* (viz. strain DC 3) which possessed a H^+-ATPase with diminished sensitivity both to *N,N'*-dicyclohexylcarbodiimide and to butyricin 7423, exhibited a negligible decrease in $\Delta\psi$ and in K^+ accumulation ratio in response to concentrations of butyricin 7423 that were bactericidal to the wild-type, parent organism. Even so, the bactericidal action of butyricin 7423 on *Cl. pasteurianum* is not adequately explained by its ability *in vitro* to inhibit the membrane H^+-ATPase of this organism.

6. Bactericidal concentrations of butyricin 7423 neither provoked efflux of Na^+ ions from *Cl. pasteurianum* nor exhibited any protonophorous activity. However, at artificially high concentration, butyricin 7423 catalysed the passage of Na^+ ions as well as of K^+ ions through multilayer lipid membranes.

7. As a non-protonophorous uncoupler, butyricin 7423 appears to act in a similar manner to that of the membrane-active colicins. Yet no evidence was obtained that butyricin 7423 at its minimum lethal concentration might form a gated ion channel in the cytoplasmic membrane of the target cell, or act as a classic ionophore.

The bacteriocins are a heterogeneous group of bactericidal proteins secreted by a variety of bacteria [1–5]. For a number of bacteriocins it has been clearly shown that the physiological target site is located within the cytoplasmic membrane of sensitive cells, and it is generally assumed that all such 'membrane-active' bacteriocins may exhibit a similar type of action upon their organisms, each dissipating the 'energised state' of the energy-coupling cytoplasmic membrane. Of all the membrane-active bacteriocins the most intensively studied have been the colicins E1, Ia and K and it has been concluded that they form ion-permeable channels in the cytoplasmic membrane [3].

The utility of membrane-active bacteriocins in investigations of bacterial membrane energy-transduction processes has been pointed out by several workers [6], and we have recently reviewed the salient features of the action of the membrane-active colicins from this standpoint [7], concluding that their key target might lie within a 'protoneural network' of membrane-located proteins.

In contrast to aerobically grown *Escherichia coli*, *Clostridium pasteurianum* is believed to possess only a single mechanism for generating an energised membrane state, namely by the protonmotive hydrolysis of ATP [8–10], and evidence has been presented elsewhere that the membrane-active bacteriocin butyricin 7423 can act as BF_0-directed inhibitor of the membrane H^+-ATPase of this organism [9]. Since it has been shown that membrane-active colicins are capable of dissipating the bulk-phase transmembrane electrical potential in sensitive cells of *E. coli* [11–14], we have studied the effects of butyricin 7423 and other compounds on the magnitude of this potential in *Cl. pasteurianum*, using the well-known phosphonium ion distribution method and a multi-ion monitoring system.

In this paper we report that whilst butyricin 7423 is indeed capable of dissipating the bulk-phase transmembrane electrical potential in *Cl. pasteurianum*, certain features of its action

Abbreviations. $BuPh_3P^+$, butyltriphenylphosphonium cation: $\Delta\psi$, apparent transmembrane, bulk-phase potential.

Enzymes. H^+-ATPase or proton-translocating adenosine 5′-triphosphatase (EC 3.6.1.3); carbonic anhydrase (EC 4.2.1.1.); granulose synthase or ADPglucose: α-1,4-glucan 4-glucosyltransferase (EC 2.4.1.21); lysozyme (EC 3.2.1.17).

suggest that neither its inhibition of the H^+-ATPase nor its effect upon the bulk-phase electrical potential can alone adequately account for its bactericidal action.

MATERIALS AND METHODS

Maintenance and Growth of Clostridium pasteurianum

The maintenance of *Cl. pasteurianum* 6013 (i.e. W-5, ATCC 6013) has been previously described [9]. In the present study we also used two previously described mutant strains of *Cl. pasteurianum* 6013. Mutant strain MR505 [15] lacked a functional granulose synthase and was asporogenous in normal glucose minimal medium; it was maintained as previously described [15]. Mutant strain DC3 exhibited diminished sensitivity to *N,N'*-dicyclohexylcarbodiimide and in glucose minimal medium was less sensitive than the parent organism to butyricin 7423 [16]; it was routinely grown and maintained in the presence of fresh dicyclohexylcarbodiimide (80 μM).

Batch cultures of strains 6013 and DC3 were grown at 37 °C under an atmosphere of N_2 plus CO_2 (19:1, v/v) in the previously described glucose, ammonium, salts and vitamins minimal medium [9]. Mutant strain MR505 was grown in a 1-l glucose-limited chemostat culture, as described [17] except that the concentration of K^+ in the inflowing medium was 2 mM. The steady-state cell density was 2.8×10^8 organisms ml^{-1} (0.38 mg dry wt ml^{-1}).

Preparation of Cell Suspensions

Samples (240 ml) were taken from the chemostat culture (in the case of strain MR505) or, in the case of strains 6013 and DC3, from batch cultures which had attained their mid-exponential phase of growth (approx. 7×10^7 organisms ml^{-1}). These were centrifuged ($5000 \times g$ for 2 min at room temperature) and the cell pellet was washed in the appropriate anaerobic and reduced medium or, as was more usually the case, was immediately resuspended in the necessary incubation mixture. Routinely, cell pellets were resuspended in 0.5 ml of incubation mixture by gentle agitation with the end of a syringe needle, followed by passage through the same needle. The resulting suspension (approx. 1 ml) was immediately injected into the reaction vessel (final reaction volume 40 ml). This procedure was normally completed within 3 min of removing the culture sample from the growth vessel. Thus cell suspensions were never stored for any significant length of time. In most cases organisms were resuspended in 50 mM triethanolamine phosphate pH 6.5 containing 0.05% L-cysteine hydrochloride, supplemented, where noted, with 0.1% D-glucose.

Ion-Selective Electrodes

The pH of cell suspensions was monitored with a glass electrode (Russell pH, Auchtermuchty, Scotland). K^+ activities were monitored with a NH_4^+/K^+-sensitive glass electrode (EIL, Chertsey, England) or with a valinomycin-based polyvinylchloride (PVC) membrane electrode, see [18], the membrane being constructed by dissolving 10 mg valinomycin in 1 ml of tetrahydrofuran and mixing this with 10 ml of 5% (w/v) polyvinylchloride (high molecular weight form) and 0.5 ml dibutyl sebacate (plasticiser). The mixture was poured into an 8.5-cm-diameter glass petri dish and the solvent allowed to evaporate at room temperature through a layer of Whatman No 1 filter paper. The ion-sensing membrane thus fabricated could be stored for months at room temperature. A lipid soluble cation (e.g. $BuPh_3P^+$) sensing membrane was constructed in a similar fashion using sodium tetraphenyl borate as ligand and dioctyl phthalate as plasticiser (cf. [19, 20]).

Potentiometric Monitoring System

The ion-selective electrodes were held in the stirred bacterial suspension which was maintained at 37 °C under oxygen-free nitrogen in an electrically grounded reaction vessel (5 ml, 50 ml or 200 ml as required). The potential differences between each of the 'working' ion-selective electrodes and a single Ag/AgCl double junction reference electrode (Orion, model 90-02) were virtually continuously monitored and recorded using a multichannel digital voltmeter interfaced to a serial drive scanner unit (model 1051, Datron Data Systems Ltd, Norwich, UK). Data acquisition and control of these devices was accomplished by means of a 32 kilobyte microcomputer (Commodore, PET), the interface bus being of the IEEE-488 (1975) type. The other devices on the bus were a dot-matrix printer, a dual-drive floppy disc system and an eight-channel digital-to-analogue converter driving a multichannel potentiometric chart recorder. The software dealing with the management and data handling of this system was written in BASIC. A more complete description of this multichannel potentiometric system is to be given elsewhere [21], together with an account of its automated calibration of each ion-selective electrode and of the manner in which it converted the raw data (in mV) from each electrode into ion activities corrected for electrode interference.

Performance of Potentiometric Experiments

Resuspension medium was added to the reaction vessel through the head space of which was passed a slow stream of oxygen-free nitrogen. Ion activities were adjusted to the desired levels (providing a useful check on the performance of the electrode calibration routines) and serial scanning of each electrode was initiated. Organisms resuspended in a small volume of reaction medium, as described above, were added to the contents of the vessel, either after a scanning (software) interrupt if the event was to be logged, or during scanning if a brief interrupt was not desired. When an interrupt was initiated, interim ion activity data together with details of changes in ion activities since the previous interrupt was directed to the printer; further additions were logged in this way. Data was continuously output to the chart recorder. There was also provision for holding any given ion activity constant by means of titrant addition. Analogue data were scaled by an interactive subroutine which could be called at any point during an experiment and which also permitted rescaling and back-off to be performed if changes in ionic activity exceeded those anticipated.

Although dicyclohexylcarbodiimide, valinomycin, gramicidin D and efrapeptin were all added from ethanolic stock solutions, each addition represented at least a 1000-fold dilution of the stock solution. At the end of each experiment with ionophores the reaction vessel was washed twice with distilled water, six times with 60% ethanol and thrice again with distilled water before fresh medium was added for pre-equilibration.

Assay and Purification of Butyricin 7423

The purification of butyricin 7423 from the supernatants of batch cultures of *Clostridium butyricum* NCIB 7423 and its bioassay (in AU) were carried out essentially as described

previously [22]. All preparations were stored under liquid nitrogen, were freshly thawed for use and were not again refrozen, any unused material being discarded. Preparations of the bacteriocin were stored at 4 °C for periods not exceeding 8 h during any series of experiments.

Flow Dialysis Linked to Atomic Emission Spectroscopy

A flow dialysis cell of the type described previously [23] was used throughout. Distilled water was pumped through the lower compartment using the vacuum pump of the atomic emission spectrometer (Pye Unicam, Cambridge, UK) at a rate ($4-5$ ml min^{-1}) depending upon the bore of the tubing employed. The output from the spectrometer was linked directly to a potentiometric chart recorder (it not being necessary to use an integrator). The system was calibrated by the addition of known quantities of KCl or NaCl to a 1-ml reaction mixture held in the upper chamber of the flow dialysis cell. The cell was held at 37 °C by means of a thermostatted water jacket.

Organisms were added as a concentrated suspension (1 ml) to the upper chamber of the flow dialysis cell which was fitted with a lid containing narrow addition ports through which was passed a continuous stream of oxygen-free nitrogen. The rate and extent of transmembrane ion flux could be measured by this means with almost the same accuracy as that obtained with the potentiometric procedure.

Preparation of Lipid-Impregnated Polycarbonate Membranes and Measurement of Ion Fluxes through Them

Nuclepore membranes (0.1-μm pore size, 13 mm diameter; Sterilin Ltd, Teddington, UK) were soaked for 30 min at 37 °C in a solution of egg lecithin (Lipid Products, Nutfield, UK) in 2-propanol. The concentration of egg lecithin employed determined the eventual thickness of the multilayer lipid membrane. The membranes were removed and dried under slight vacuum and then conditioned overnight in 50 ml of the appropriate incubation mixture (cf. [24]). This was either 0.1 M potassium phosphate pH 6.6, or 0.1 M Tris/HCl pH 7.2. The flow dialysis cell described above was used to trap the lipid-impregnated membrane between two aqueous compartments, water tightness being ensured by the use of PTFE (polytetrafluoroethylene) gaskets, the rate of flux of an ion through the membrane thereafter being followed by the usual flow dialysis procedure. Na^+ and K^+ fluxes were determined using the on-line atomic emission spectrometric system outlined above. The detection system was calibrated by the passage through it of solutions of known composition prior to an experimental run.

Determination of Protein

Protein was measured by the Folin method [25] with bovine serum albumin (fraction V) as standard. Bacterial cells were first autoclaved in 0.1 M NaOH and centrifuged for 3 min at $5000 \times g$ prior to protein determination so as to avoid interference by the cysteine usually present in their suspensions.

Determination of Intracellular Volume

This was performed exactly as described elsewhere [23] using lactose as the membrane-impermeant species. The value obtained was used in the calculation of the membrane potential, with the usual assumptions and formulae [20, 23].

Chemicals

Dicyclohexylcarbodiimide, gramicidin D, monactin, sodium tetraphenylborate and valinomycin were obtained from the Sigma (London) Chemical Co. (Poole, UK). Butyltriphenylphosphonium (bromide salt) was from Koch-Light Laboratories (Colnbrook, UK). PVC, dioctyl phthalate, dibutyl sebacate and triphenylmethylphosphonium bromide were from Aldrich Chemical Co. (Poole, UK). Efrapeptin was the generous gift of Prof. R. B. Beechey (Shell Research Ltd, Sittingbourne, UK). All other chemicals were obtained from commercial sources, generally BDH Chemicals Ltd (Poole, UK), and were of analytical grade or of the highest purity available.

RESULTS

Optimisation and Critical Evaluation of Experimental Conditions

Concentrated suspensions of *Clostridium pasteurianum* strain MR505 were freshly prepared from samples (240 ml) removed from a glucose-limited chemostat culture ($A_{680}^{1\,cm} = 0.95$). These organisms had been grown in the presence of 2 mM K^+ and estimations of their intracellular volume revealed that the lactose-impermeable space was 1.4 μl mg dry $weight^{-1}$ (0.026 μl per 10^7 organisms). A number of such estimations were routinely made to take account of any week by week changes, but none was apparent during the course of the present experiments.

When these organisms were resuspended in 1 ml of minimal resuspension medium (i.e. 50 mM triethanolamine phosphate) supplemented with 0.05% cysteine hydrochloride prior to injection into the anaerobic reaction vessel, a measurable amount of intracellular K^+ had already leaked from the cells, as was evidenced by the significant 'step' increase in extracellular K^+ activity observed at addition of the cell suspension in Fig. 1. This K^+ leakage continued until glucose (0.1%) was added to the anaerobic suspension (Fig. 1b). If this K^+-leaky bacterial suspension was incubated for longer than approximately $20-25$ min in the absence of glucose, no more intracellular K^+ could be encouraged to efflux from the cells by the addition of valinomycin plus nigericin (Fig. 1a). Furthermore, when the leakage of K^+ due to the absence of an energy source was more than 90% complete, the organisms were unable to reinitiate glycolysis and re-accumulate effluxed K^+ (Fig. 1a).

A short time, usually less than $1-2$ min, after addition of glucose to organisms leaking K^+ into a medium already containing $2-3$ mM K^+ (Fig. 1b), the net efflux of K^+ ceased and was replaced by extensive net accumulation of K^+ from the extracellular medium. At this time glycolysis, monitored either by the decrease in the pH of the suspension (Fig. 1) or as acetate production (Fig. 2), recommenced (Fig. 1b). The net uptake of K^+ continued until it abruptly ceased when the organisms had achieved a steady state in which no net efflux or influx of K^+ could be measured (± 5 μM). This steady state was sustained for as long as 1 h, as was a relatively constant rate of glycolysis. Addition of valinomycin to non-glycolysing cells accelerated their rate of loss of K^+ ions and abbreviated the period wherein glycolysis could be reinitiated by the addition of glucose (Fig. 1c). That butyricin 7423 did not have a simple valinomycin-like effect was evidenced by the fact that its addition to non-glycolysing cells neither stimulated the rate of K^+ efflux (Fig. 1d) nor caused a K^+ diffusion potential.

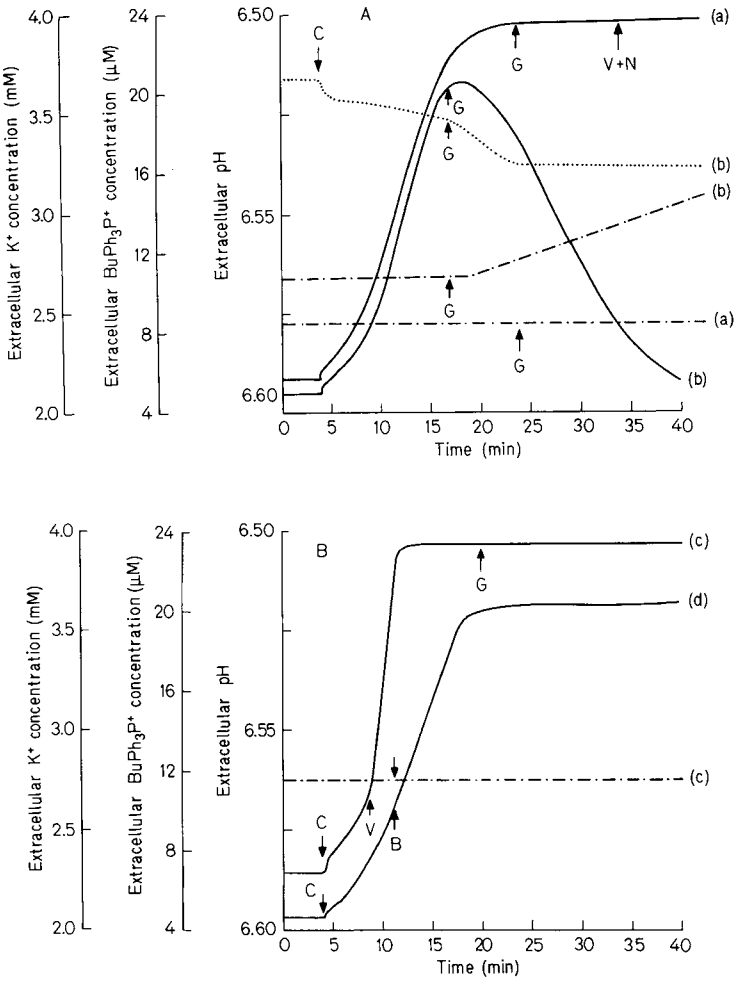

Fig. 1. *Membrane potential and K^+ uptake by* Cl. pasteurianum; *interplay with glycolysis.* Cells of *Cl. pasteurianum* MR505 (6.7×10^{10} organisms) were added at the time marked C to a 40-ml reaction mixture containing 50 mM triethanolamine phosphate pH 6.55–6.60, 2–2.5 mM KCl and 21.5 μM $BuPh_3P^+$. Ionic fluxes were monitored as described in Materials and Methods. Butyricin 7423 (B) at 1 AU per 10^7 organisms, glucose (G) at 0.1%, valinomycin (V) at 1 μg ml^{-1}, and nigericin (N) at 1 μg ml^{-1} were added at the times indicated. (A) (a) Nil restoration of glycolysis by glucose addition following complete efflux of K^+ from cells deprived of glucose. (b) Restoration of glycolysis by glucose addition following incomplete efflux of K^+ from similar cells. (B) (c) Accelerated efflux of intracellular K^+ ions provoked by valinomycin. (d) Exposure of glucose-deprived cells to butyricin 7423 caused no change in the rate of efflux of intracellular K^+ ions. Key: Extracellular K^+ concentration (———), extracellular $BuPh_3P^+$ concentration (·····), extracellular pH, with H^+ production reflecting glycolysis (–·–·–·–)

A relatively low concentration of the membrane-permeable cation butyltriphenylphosphonium ($BuPh_3P^+$) was employed in these experiments for three reasons. Firstly, high extracellular concentrations of $BuPh_3P^+$ would tend to lower the observed membrane potential by a significant amount. Secondly, the intracellular concentration of $BuPh_3P^+$ accumulated from a high extracellular concentration of this ion might be so great as to interfere with cell metabolism. Thirdly, $BuPh_3P^+$ in excess of 100 μM was in any event growth inhibitory to *Cl. pasteurianum* when the K^+ concentration in the medium was 1–50 mM (data not shown). The concentration (approx. 20 μM) of $BuPh_3P^+$ which was finally employed was selected having first demonstrated that the values of $\Delta\psi$ derived from the energy-dependent distribution ratio of the cation at this concentration were identical with those values obtained using lesser concentrations (2–10 μM), indicating that artefactual depolarisation of the membrane could be discounted.

The uptake of $BuPh_3P^+$ actually proceeded in three stages in these experiments. The first was of small magnitude and occurred immediately upon the addition of non-glycolysing cells to the incubation mixture. This uptake may be ascribed to binding of $BuPh_3P^+$ to sites in and around the cell surface and was equal to the extent of binding observed with completely uncoupled organisms (data not shown). Whilst the organisms continued to be incubated in the absence of glucose a further, slow phase of $BuPh_3P^+$ uptake was evident. This uptake coincided with the leakage of K^+ from the cells (Fig. 1, 2) and it is therefore likely that the leakage of K^+ from non-glycolysing cells generated a diffusion potential to which $BuPh_3P^+$ could respond (cf. [26]). On addition of glucose and reinitiation of glycolysis, a further uptake of $BuPh_3P^+$ was observed in concert with energy-linked uptake of K^+ ions. The accumulation of $BuPh_3P^+$ reached a steady state long before the net accumulation of K^+ was complete (Fig. 1b), and remained in this steady state for as long as 1 h.

When valinomycin (0.5 μg ml^{-1}) was added to suspensions which had achieved their steady state (zero net K^+ uptake) a significant proportion of intracellular K^+ effluxed from the cells. This generated a diffusion potential (negative inside) and

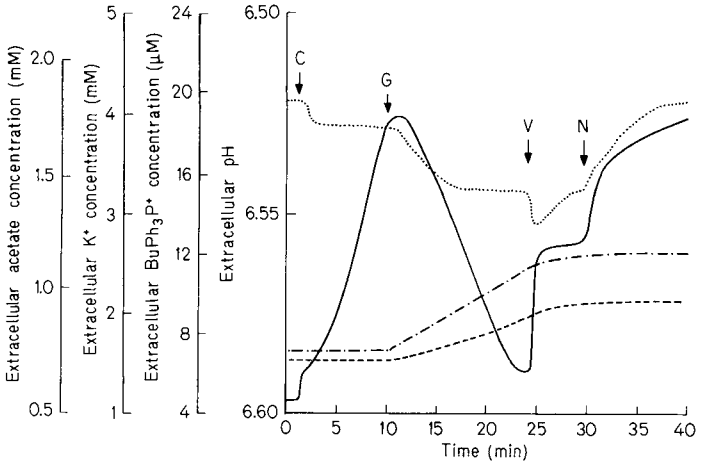

Fig. 2. *Effect of valinomycin and nigericin on K^+ accumulation and on the membrane potential of* Cl. pasteurianum *at a low extracellular concentration of K^+*. Cells of *Cl. pasteurianum* MR 505 (6.7×10^{10} organisms, 91.2 mg dry weight) were added at the time marked C to the same reaction mixture as described in the legend to Fig. 1. Ionic fluxes were monitored as described in Materials and Methods. Additions were as in Fig. 1. Key: Extracellular K^+ concentration (——), extracellular $BuPh_3P^+$ concentration (·····), extracellular pH (–·–·–·–), extracellular acetate concentration (– – – –)

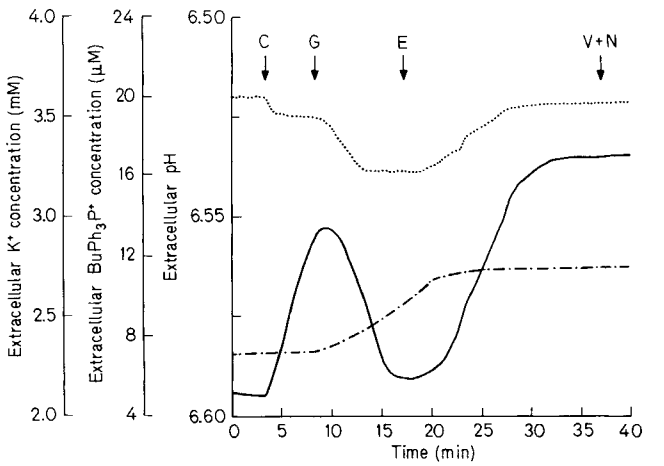

Fig. 3. *Effect of the membrane H^+-ATPase inhibitor efrapeptin on ionic fluxes in* Cl. pasteurianum. The experiment was performed as described in the legend to Fig. 1 using cells of *Cl. pasteurianum* MR 505. Efrapeptin (E) was added at the time indicated, to a final concentration of 0.8 μg ml^{-1}. Other additions and symbols as in Fig. 1

$BuPh_3P^+$ ions were rapidly accumulated (Fig. 2). The observation that $BuPh_3P^+$ accumulation in response to a very rapid efflux of K^+ was also rapid, showed that the rate of passage of $BuPh_3P^+$ through the cell membrane was not limiting in the calculation of $\Delta\psi$. Thus under most conditions the response of the $BuPh_3P^+$-electrode and the rate of equilibration of $BuPh_3P^+$ across the cell membrane in response to $\Delta\psi$ were sufficiently rapid to provide a good, continuous measure of the transmembrane potential. The steady-state membrane potential was calculated [20, 23] from the accumulation ratio of $BuPh_3P^+$ using the value obtained for the lactose-impermeable space as the available intracellular volume, and was found to be 100–110 mV prior to the addition of valinomycin (Fig. 1, 2). The addition of valinomycin plus nigericin should catalyse total efflux of actively accumulated K^+ ions and therefore permit one to determine the total (bound plus free) intracellular K^+ content. In the above experiment the total intracellular K^+ concentration was approximately 390 mM in glycolysing organisms under static head conditions, which in terms of a steady state electrogenic accumulation ratio would be in equilibrium with a $\Delta\psi$ of 134 mV.

Effects of Inhibitors of H^+-ATPase on the Accumulation of $BuPh_3P^+$ and K^+ Ions by Cl. pasteurianum

The BF_1-directed membrane H^+-ATPase inhibitor efrapeptin and the BF_0-directed H^+-ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide were added to glycolysing cell suspensions which had achieved the above steady state. These inhibitors provoked a slow efflux of both $BuPh_3P^+$ and K^+ ions, and the effect of efrapeptin (0.8 μg ml^{-1}; Fig. 3) was similar to that of dicyclohexylcarbodiimide. Glycolysis was inhibited and both $BuPh_3P^+$ and K^+ ions effluxed until their transmembrane gradients had been abolished, as evidenced by the fact that subsequent co-addition of valinomycin and nigericin provoked no further efflux of these ions. It should be noted that this inhibition of glycolysis was not a consequence of the loss of intracellular K^+ and is ascribed, directly or indirectly, to the accumulation of ATP (cf. [16]) since this inhibition was greatly delayed by the substitution of arsenate for phosphate in the reaction mixture (data not shown). Efrapeptin had similar effects on the mutant strain DC3 which possesses a membrane H^+-ATPase which is resistant to both dicyclohexylcarbodiimide and butyricin 7423. It may be concluded that ATP hydrolysis catalysed by the H^+-ATPase(BF_0F_1) represents the sole mechanism for generating a membrane potential in this organism.

Effect of Butyricin 7423 on Accumulation of $BuPh_3P^+$ and K^+ Ions

The addition of an excess of butyricin 7423 (5 AU per 10^7 organisms) to glycolysing suspensions of *Cl. pasteurianum* which had achieved a steady state of K^+ and $BuPh_3P^+$ uptake (Fig. 4A) provoked an immediate and continuing efflux of both K^+ and $BuPh_3P^+$ ions. The rate of K^+ efflux was slower than that elicited by valinomycin (Fig. 2) but the butyricin-provoked efflux of these ions was complete since no further efflux could be evoked by the addition of valinomycin plus nigericin. The stepwise addition of lower concentrations of butyricin 7423 to similar suspensions of organisms provoked the efflux of discrete quantities of K^+ ions rather then the steady efflux observed at higher concentrations of the bacteriocin (Fig. 4B). This was not accompanied initially by any decline in the measured accumulation of $BuPh_3P^+$. However, the accumulation ratio of $BuPh_3P^+$ eventually began to decline and continuing efflux of K^+ ions supervened; by this time glycolysis had ceased (Fig. 4B).

It had previously been noted that butyricin 7423 provoked even more rapid efflux of preloaded $^{86}Rb^+$ ions when the organisms were suspended in an isotonic buffer mixture essentially free of K^+ ions [27]. Therefore an experiment similar to that described above was performed using 15% (w/v) sucrose-supplemented, 50 mM sodium phosphate buffer pH 7.0 as the incubation medium. On addition of a slight excess of butyricin 7423 a much more rapid rate of efflux of K^+ ions was observed (Fig. 5b) than had been provoked by the same concentration of the bacteriocin under the previous reaction conditions (Fig. 5a). This efflux was again complete.

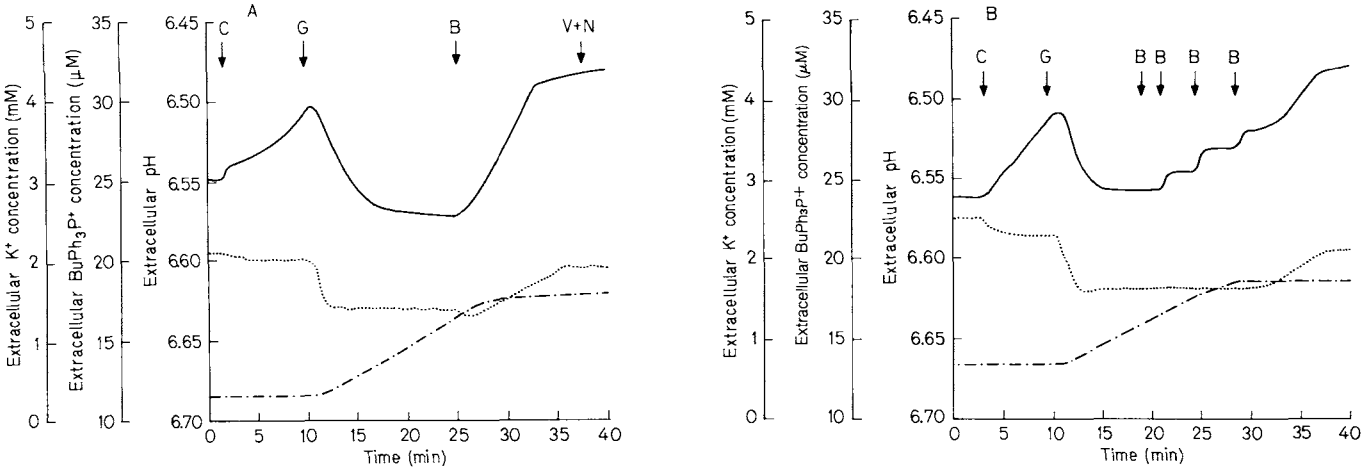

Fig. 4. *Effect of butyricin 7423 on K^+ accumulation and membrane potential in* Cl. pasteurianum *MR505*. The experiment was performed as described in the legend to Fig. 1. Butyricin 7423 (B) was added as indicated, in to a final concentration of 5 AU per 10^7 organisms, and in B sequentially at 0.2, 0.8, 1.0 and 2.0 AU per 10^7 organisms. Other additions and symbols as in Fig. 1

To separate the inhibitory effects upon glycolysis of (i) K^+ loss, and (ii) increase in intracellular ATP, both of which events were elicited by the addition to *Cl. pasteurianum* of efrapeptin or of excess butyricin 7423, similarly grown organisms were suspended in media containing a higher concentration of K^+ ions (approximately 18 mM). At such external K^+ concentrations the joint addition of valinomycin plus nigericin had relatively little effect upon their rate of glycolysis (Fig. 6a). However, treatment of similar cell suspensions with efrapeptin still inhibited glycolysis, providing further evidence that inhibition of glycolysis by this ATPase inhibitor was not primarily a result of loss of intracellular K^+ (Fig. 6b). In this respect butyricin 7423 acted similarly to efrapeptin (Fig. 6c). It is noteworthy that under no conditions in which butyricin 7423 provoked net efflux of K^+ ions was a diffusion potential established similar to that evinced by valinomycin, suggesting that butyricin 7423 cannot be a simple K^+-ionophore.

Inability of Butyricin 7423 to Provoke K^+ Efflux from Non-glycolysing Cells of Cl. pasteurianum

When *Cl. pasteurianum* was deprived of glucose, a substantial rate of K^+ efflux occurred which was accompanied by an uptake of $BuPh_3P^+$ (Fig. 1). This rate of K^+ efflux could be increased by the addition of valinomycin (or nigericin) but not by an excess of butyricin 7423 (Fig. 1c, d).

It is difficult to say whether this represents a situation in which the organisms are essentially de-energised, and in which it is therefore to be expected that membrane-active bacteriocins should not interact with the cytoplasmic membrane of sensitive cells; under the described conditions there remained a substantial diffusion potential across the bacterial cell membrane which should, in principle, have provided a sufficient bulk-phase $\Delta\psi$ to promote any $\Delta\psi$-dependent 'gating' phenomenon as suggested elsewhere [28], but cf. [7, 29].

K^+ Ion Flux Provoked by Butyricin 7423 in Cl. pasteurianum *Mutant DC3*

Since mutant strain DC3 had already been shown to possess a membrane H^+-ATPase displaying diminished sensitivity to inhibition both by dicyclohexylcarbodiimide and butyricin 7423 [16], it was of interest to establish whether butyricin 7423

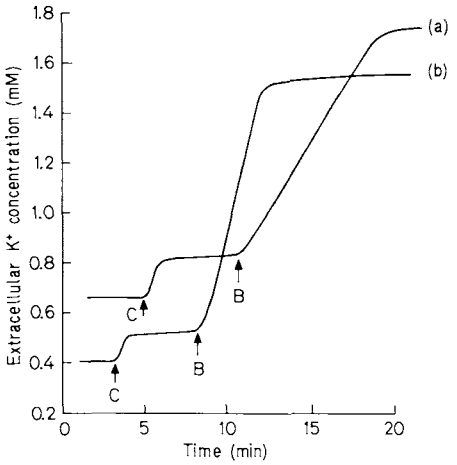

Fig. 5. *Rates of K^+ efflux from* Cl. pasteurianum *provoked by butyricin 7423 at very low extracellular concentrations of K^+*. The experiment was performed in the manner described in Materials and Methods using *Cl. pasteurianum* MR505 at a final cell concentration of 2.3 mg dry weight ml^{-1}. The reaction mixtures contained: (a) 50 mM triethanolamine phosphate pH 6.55, 0.05% cysteine hydrochloride, 0.1% glucose and 0.65 mM K^+; (b) 50 mM sodium phosphate pH 7.0 plus 15% (w/v) sucrose and 0.4 mM K^+. Cells (C) and butyricin 7423 (B) at 1 AU per 10^7 organisms were added at the times indicated in each case

exerted any effect upon the membrane potential of this organism. Organisms for these experiments were grown in 2% (w/v) glucose minimal medium containing 50 mM sodium phosphate pH 7.0, 2 mM KCl and 80 μM dicyclohexylcarbodiimide and were harvested in the mid-exponential phase of batch growth (approximately 7×10^7 organisms ml^{-1}) well before granulose deposition was microscopically visible upon staining with iodine. This mutant strain behaved similarly to the parental, wild-type strain after resuspension and addition of 0.1% glucose (Fig. 7). The addition of butyricin 7423 to such suspensions when they had achieved the normal steady state provoked a small efflux of K^+ ions. This net efflux was however quickly negated by an equivalent amount of reuptake of K^+ ions and the re-establishment of the original steady state of K^+ accumulation (Fig. 7). Subsequent addition of an excess of butyricin 7423 provoked a barely-measurable efflux of K^+ and

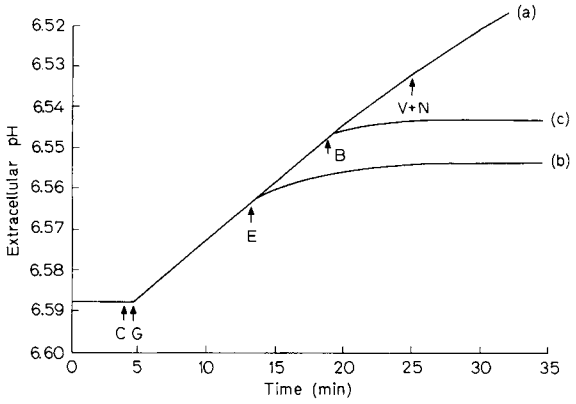

Fig. 6. *Effect of ionophores and inhibitors of H^+-ATPase on glycolysis by* Cl. pasteurianum *MR505 at an extracellular K^+ concentration of 18 mM.* Experiments were performed as described in the legend to Fig. 1 save for the increased extracellular concentration of K^+. Additions were: C, cells (2.3 mg dry weight ml^{-1}) and G, glucose (0.1%) in all traces; V, valinomycin at 1 μg ml^{-1} plus N, nigericin at 1 μg ml^{-1} (trace a); E, efrapeptin at 0.8 μg ml^{-1} (trace b); B, butyricin 7423 at 4 AU per 10^7 organisms (trace c)

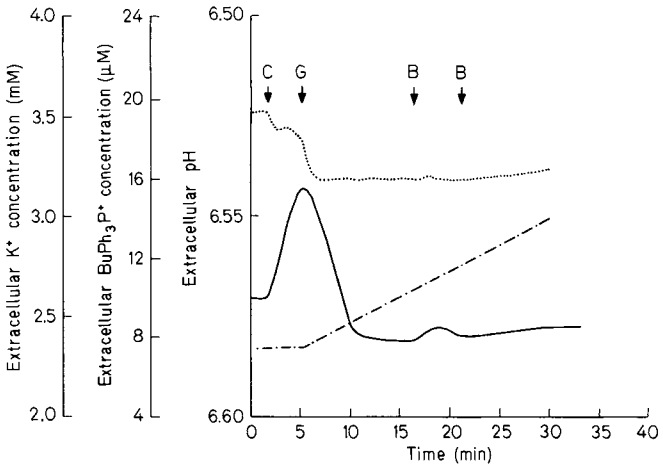

Fig. 7. *Effect of butyricin 7423 on ion fluxes in* Cl. pasteurianum *mutant strain DC3.* The experiment was performed as described in the legend to Fig. 1. The final cell concentration in the reaction vessel was approximately 2 mg dry weight/ml. Butyricin 7423 (B) was added, at the times indicated, to final concentrations of 2 and 8 AU per 10^7 organisms

$BuPh_3P^+$ ions. Glycolysis was unaffected throughout this experiment.

Negligible Efflux of Sodium Ions Provoked by Butyricin 7423

Flow dialysis with effluent monitoring by atomic emission spectroscopy was used to measure both Na^+ and K^+ fluxes in cell suspensions of *Cl. pasteurianum.* Organisms were added to the upper compartment of a flow dialysis cell (Materials and Methods) in the presence of 100 mM triethanolamine phosphate pH 7.0 containing 0.05% cysteine and 0.1% glucose whilst oxygen-free nitrogen was passed over the surface of the liquid in this compartment. When the suspension had achieved a state of nil net uptake or efflux of both Na^+ and K^+, butyricin 7423 was added. As observed in the potentiometric assay, when butyricin 7423 was added at a relatively low concentration it provoked a discrete efflux of K^+ ions and the organisms established a new steady state of K^+ content (Fig. 8). Efflux of Na^+ ions could not be provoked by similar concentrations of the bacteriocin; even when a great excess of butyricin 7423 was employed, only a very small efflux of Na^+ was observed (Fig. 8). Gramicidin D provoked efflux of both Na^+ and K^+ ions until their energy-linked concentration gradients were discharged.

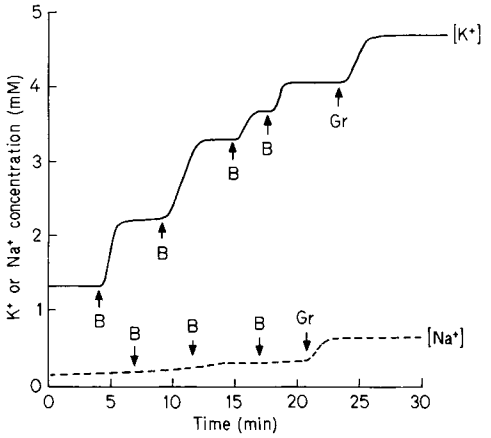

Fig. 8. *Effect of butyricin 7423 on K^+ and Na^+ efflux from intact cells of* Cl. pasteurianum. K^+ and Na^+ fluxes were monitored by flow dialysis linked to atomic emission spectroscopy as described in Materials and Methods. The upper compartment of the flow dialysis cell contained, in a final volume of 1 ml, 50 mM triethanolamine phosphate pH 6.55, 0.1% glucose, 0.05% cysteine hydrochloride, cells of *Cl. pasteurianum* MR505 equivalent to a dry weight of 5 mg, plus sufficient K^+ (as KCl) and Na^+ (as NaCl) to give the initial effluent concentrations indicated. The traces have been linearised to remove the effect of the very slow loss of ions from the upper compartment of the flow dialysis cell. In the case of the K^+ trace, butyricin 7423 was added, as indicated, to final concentrations of 0.8, 1.8, 2.8 and 3.8 AU per 10^7 organisms. In the Na^+ trace, butyricin 7423 was added at the times indicated (B) to final concentrations of 1,3 and 5 AU per 10^7 organisms. Gramicidin D (Gr) was added, at the times indicated, to a final concentration of 5 μg ml^{-1}

Nil Effect of Butyricin 7423 on the Proton Permeability of the Cell Membrane

It is not possible to estimate the H^+ permeability of the cell membrane of glycolysing *Cl. pasteurianum* since large quantities of acetate and butyrate are produced which confound any sensitive measurement of pH changes. For this reason, energy-starved organisms were employed. The experimental design was similar to that used by Harold and Baarda [30] with *Streptococcus faecalis.* Glucose-deprived cells were prepared by suspending organisms in 50 mM sodium phosphate pH 7.0 containing 1 mM KCl and 2% lactose, followed by anaerobic incubation at 37 °C for 4 h to starve the organisms. After this period, the organisms were reharvested and suspended (at 1 mg dry weight ml^{-1}) in 1 mM glycylglycine hydrochloride pH 6.8 containing 6 mM $MgSO_4$, 1 mM 2-mercaptoethanol and 50 mM KCl. Portions (4 ml) of this suspension were introduced into a vessel held at 37 °C and through whose head space was passed a stream of oxygen-free nitrogen. Carbonic anhydrase (40 μg) was added and the suspension was allowed to equilibrate until a steady pH trace was obtained. After about 15 min the suspension was adjusted to pH 6.2 by the addition of HCl. Addition of valinomycin (1 μg ml^{-1}) then provoked an increase in the rate of proton ingress into the cells and this rate

could be further enhanced by the addition of tetrachlorosalicylanilide (Fig. 9). The order of addition of these ionophores did not affect the eventual rate of H^+ uptake (Fig. 9).

Addition of butyricin 7423 (1 AU per 10^7 organisms) before or after the addition of valinomycin did not affect the rate of decay of the artificially created transmembrane pH gradient. However, butyricin 7423 did exert a valinomycin-like, or detergent-like, effect (Fig. 9) at unphysiologically high concentrations (10 AU per 10^7 organisms). In no case was there any evidence to suggestst that butyricin 7423 acted as a protonophore.

Effect of Butyricin 7423 on $^{86}Rb^+ - K^+$ Exchange

The K^+ efflux that is provoked by butyricin 7423 could conceivably be the consequence of any of a number of events:

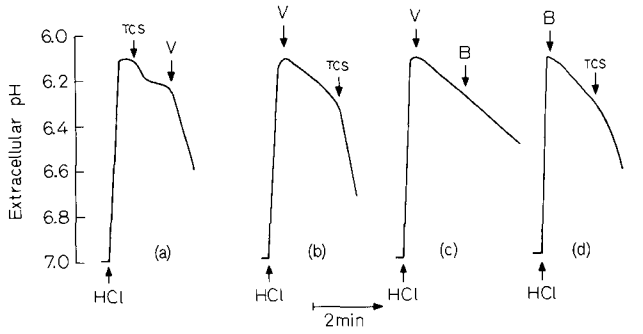

Fig. 9. *Nil effect of butyricin 7423 on the proton permeability of intact cells of* Cl. pasteurianum. Suspensions of starved cells of strains MR 505 were prepared as described in the text. Acid (HCl) pulses were given at the outset, followed by additions, as indicated, of: (a) tetrachlorosalicylanilide (TCS) at 1 μM, valinomycin (V) at 1 μg ml^{-1}; (b) Valinomycin (V) at 1 μg ml^{-1}, tetrachlorosalicylanilide at 1 μM; (c) Valinomycin (V) at 1 μg ml^{-1}, butyricin 7423 (B) at 1 AU per 10^7 organisms; (d) Butyricin 7423 (B) at 1 AU per 10^7 organisms, tetrachlorosalicylanilide (TCS) at 1 μM

(a) inhibition of membrane H^+-ATPase, (b) formation of an 'ion pore' in the membrane, (c) uncoupling of energy supply to the K^+ porter. Previous evidence [9, 27] suggested that although the first mechanism might be a prominent component of butyricin action, a more direct mechanism might be promoting K^+ release. The following experiment was performed to resolve this question.

Organisms were allowed to attain their usual steady state of null net K^+ ion flux in the presence of 6 mM added K^+. When no net flux of K^+ (±5 μM) could be measured, 10 μCi of carrier-free $^{86}RbCl$ was rapidly injected into the well-mixed suspension. Samples (1 ml) were removed as quickly as possible and each was vacuum-filtered through a Millipore membrane (0.22-μm pore size) which was then washed with 5 ml of the resuspension mixture. The filter membrane was dried under an infrared lamp and its radioactivity measured as previously described [27]. The $^{86}Rb^+/K^+$ ratio at the addition of the radiochemical was known and thus radioactive tracer flux could be expressed as μmol equivalents of K^+ ions.

Since no net flux of K^+ ions could simultaneously be detected by the K^+-sensitive electrode, the observed rate of uptake of $^{86}Rb^+$ ions (Fig. 10) may be taken to represent the rate of K^+ ion uptake necessary to maintain intracellular levels steady in the presence of an obviously substantial rate of K^+ leakage. The result of introducing valinomycin into such suspensions immediately after addition of the $^{86}Rb^+$ clearly demonstrated that, as expected, this ionophore rapidly equilibrated $^{86}Rb^+$ across the cell membrane ($^{86}Rb^+$ influx) although there was a net K^+ efflux (Fig. 10). Butyricin 7423 was however incapable of performing such $^{86}Rb^+$ and K^+ equilibrations. Indeed, rather than promoting $^{86}Rb^+$ influx it actually inhibited it. This effect of the bacteriocin could be observed well before any effect upon K^+ efflux was measurable (Fig. 10) and was more pronounced when the experiment was performed at a lower external K^+ concentration (data not given). These findings strongly suggest that butyricin 7423 does not form an ion pore in the cell membrane.

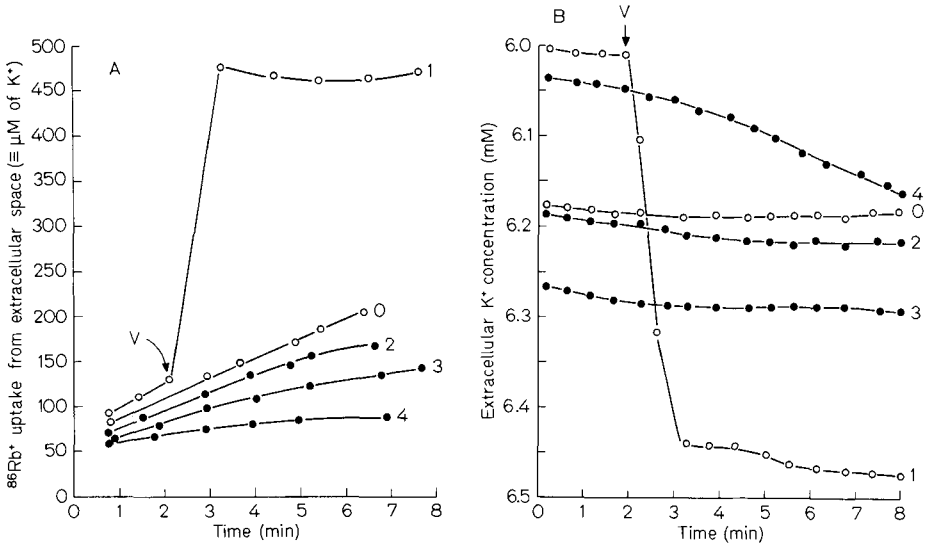

Fig. 10. *Contrasted effects of valinomycin and of butyricin 7423 on $^{86}Rb^+$ ion flux across the cell membrane of glycolysing cells of* Cl. pasteurianum. Each experiment was performed as described in the text following the attainment of a steady state of nil net flux of K^+ across the cytoplasmic membrane of glycolysing cells of *Cl. pasteurianum* MR 505. (A) The $^{86}Rb^+$ influx (expressed as the 'equivalent' K^+ exchange). (B) The simultaneous K^+ flux monitored with the K^+-sensitive electrode. At the times indicated the following additions were made: trace 0, nil addition; trace 1, valinomycin (V) at 2 μg ml^{-1}; trace 2, butyricin 7423 at 0.2 AU per 10^7 organisms; trace 3, butyricin 7423 at 0.8 AU per 10^7 organisms; trace 4, butyricin 7423 at 2 AU per 10^7 organisms. Key: no butyricin 7423 added (○); butyricin 7423 added at 0.6 min (●)

Effect of Tetrachlorosalicylanilide on K^+ Accumulation and on the Ability of Butyricin 7423 to Provoke K^+ Efflux

Tetrachlorosalicylanilide is a recognised protonophore [31] and should therefore be capable of promoting nearly complete K^+ ion efflux from *Cl. pasteurianum* since it may be expected completely to de-energise the cytoplasmic membrane. Indeed, after treatment with excess tetrachlorosalicylanilide little extra K^+ could be persuaded to efflux from the cells be the addition either of valinomycin plus nigericin or of Triton X-100 (0.1 %). The $BuPh_3P^+$ accumulation ratio also decreased as expected following addition of excess tetrachlorosalicylanilide and, as a rather late event, glycolysis ceased, presumably due in this instance to the depletion of K^+ ions from the cell. However, if discrete additions of low concentrations of tetrachlorosalicylanilide were made, a stepwise efflux of K^+ ions was obtained. This experimental system could therefore be used to assess the effect of $\Delta\psi$ on the initial rate of K^+ efflux provoked by butyricin 7423. Concentrations of tetrachlorosalicylanilide were chosen so that no more than 20 % of the total intracellular K^+ had effluxed after the addition of the protonophore. In each experiment the total concentration of intracellular K^+ ions was inferred from the total efflux provoked by addition of valinomycin (1 μg ml^{-1}) plus Triton X-100 (0.1 %). Thus it is not likely, within the limitations of this procedure, that the intracellular concentration of K^+ ions itself would so affect the initial rate of bacteriocin-provoked K^+ efflux as to obscure any specific effect of tetrachlorosalicylanilide on the response to butyricin 7423.

It is clear from the traces recorded in Fig. 11 that tetrachlorosalicylanilide markedly inhibited the rate of K^+ ion efflux provoked by butyricin 7423. With the concentrations of tetrachlorosalicylanilide used, the membrane potential was not completely dissipated and the effect of the protonophore upon butyricin-provoked K^+ efflux was essentially continuously increasing in magnitude over the range of concentrations of tetrachlorosalicylanilide employed, both with intact cells (Fig. 11) and with protoplasts (Fig. 12).

Ability of Butyricin 7423 to Increase the Ion Permeability of Planar Lipid Membrane Films

Filter membranes (Nuclepore) were used as supports for the manufacture of multilayer planar lipid membranes (Materials and Methods). The rates of efflux of $K^+$and Na^+ ions through these membranes were monitored using the flow dialysis method with atomic emission spectroscopy. In contrast to the rather specific action of bactericidal (but low) concentrations of butyricin 7423 on the membranes of whole cells of *Cl. pasteurianum*, the bacteriocin when applied at unphysiologically high concentrations was able to provoke both K^+ and Na^+ flux across these artificial planar membranes (Fig. 13). These effects occurred, of course, in the absence of any applied electrical potential across the planar lipid membrane.

DISCUSSION

The work here described has had two main purposes: firstly, to further our understanding of the physiological effects elicited by the addition of butyricin 7423 to suspensions of *Clostridium pasteurianum*, with a view to accounting for the bactericidal effect of this bacteriocin; secondly, to determine whether the interaction between the membrane-active bacteriocin and its target cytoplasmic membrane can be accomodated within our present understanding of membrane energy transduction in this organism. For the latter purpose we have made no assumptions regarding the significance of the bulk-phase protonmotive force other than it either is [32] or is not [33–35] in rapid electrochemical equilibrium with the electrochemical gradient of protons between one membrane surface and the other under energised conditions (Fig. 14).

Does the Distribution of Phosphonium Ions Adequately Reflect the Bulk-Phase $\Delta\psi$?

The strongest evidence that the phosphonium ion-distribution method may be used to give a quantitative estimate

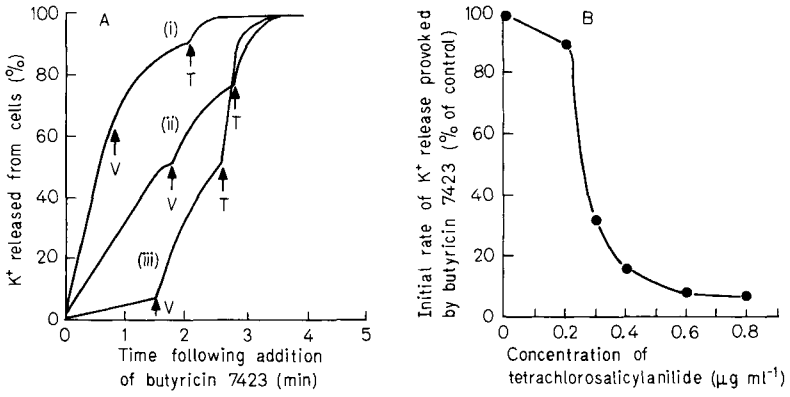

Fig. 11. *Effect of tetrachlorosalicylanilide on the rate of efflux of K^+ provoked by exposure to butyricin 7423 of intact cells of* Cl. pasteurianum. Cells of *Cl. pasteurianum* 6013 freshly harvested from mid-exponential phase batch culture were resuspended (at 4×10^9 organisms ml^{-1}) in a reaction medium containing 50 mM triethanolamine hydrochloride pH 6.5, 1 % glucose and 2 mM KCl. Tetrachlorosalicylanilide was added at the concentrations indicated, and K^+ efflux was followed with a K^+-sensitive electrode. When no further K^+ efflux was observed, butyricin 7423 was added at a low concentration (0.2 AU per 10^7 organisms) and the efflux of K^+ was again monitored. (A) Efflux of K^+ is plotted as the percentage of the total K^+ that was mobilised by Triton X-100 (T) at 0.1 %, versus time after addition of the butyricin 7423. In the three experiments recorded, tetrachlorosalicylanilide was present at concentrations (i) nil, (ii) 0.3 μg ml^{-1}, (iii) 0.6 μg ml^{-1}. Experiments performed using other concentrations of the protonophore have been omitted for reasons of clarity. A secondary plot of data obtained as in A is given in B, wherein 100 % represents a K^+ efflux rate of 600 nmol ml^{-1} min^{-1}. When employed, valinomycin (V) was added at a concentration of 1 μg ml^{-1}.

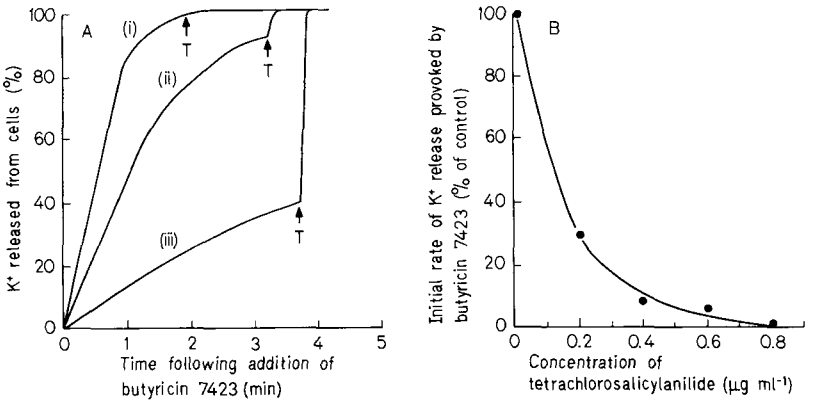

Fig. 12. *Effect of tetrachlorosalicylanilide on the rate of efflux of K^+ provoked by exposure to butyricin 7423 of protoplasts of* Cl. pasteurianum. Protoplasts were prepared by treatment with lysozyme of cells of *Cl. pasteurianum* 6013 harvested from a mid-exponential phase batch culture [8]. They were resuspended in an anaerobic buffer mixture containing 15% (w/v) lactose, 50 mM triethanolamine hydrochloride pH 6.5, 6 mM $MgSO_4$ and 10 mM 2-mercaptoethanol. Otherwise the experiments were performed, and the findings are presented, as described in the legend to Fig. 11. (A) The concentrations of tetrachlorosalicylanilide were (i) nil, (ii) 0.4 μg ml^{-1}, (iii) 0.8 μg ml^{-1}. (B) The initial rates of K^+ efflux provoked by butyricin 7423, corrected for 'background' K^+ efflux occurring in the absence of butyricin 7423, are plotted against the concentrations of tetrachlorosalicylanilide employed, 100% representing an initial butyricin-provoked K^+ efflux rate of 560 nmol K^+ ml^{-1} min^{-1}. The protoplasts were at a final concentration of 40 mg protein ml^{-1} and butyricin 7423 was added at 2 AU mg^{-1} protein

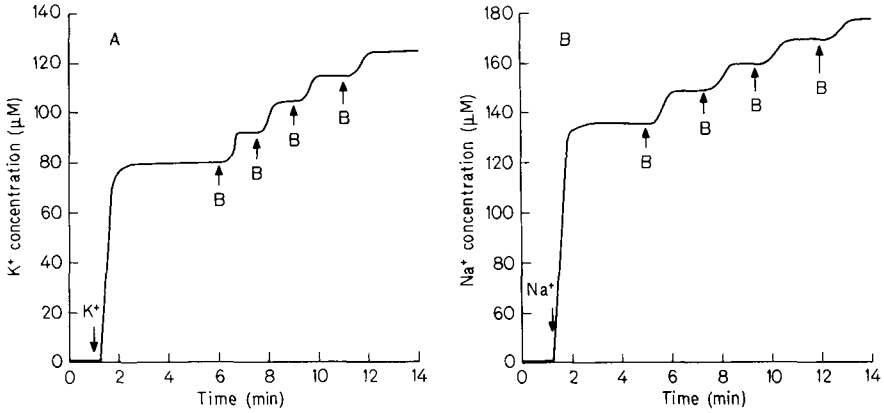

Fig. 13. *Effect of butyricin 7423 on ion fluxes across multilayer, planar lipid membranes.* Multilayer lipid membranes were prepared as described in Materials and Methods. The upper compartment of the flow dialysis cell contained 0.9 ml of 0.1 M Tris/HCl pH 7.2. (A) 0.1 ml of 1 M KCl was added at the time indicated (K^+) to the upper chamber of the flow dialysis cell, followed by four additions of butyricin 7423 (B) as indicated, to final concentrations respectively of 50, 100, 150 and 200 AU ml^{-1}. (B) The experiment was performed exactly as in A save that 0.1 ml of 1 M NaCl was added as indicated (Na^+) in place of the KCl. The experiments were performed at room temperature (approx. 22 °C)

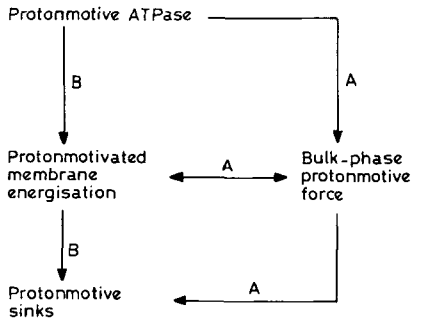

Fig. 14. *Models of membrane energy coupling in* Cl. pasteurianum. Two routes of protonmotivated membrane energy coupling are diagrammed. In the strictly chemiosmotic model (route A) the proton current is carried in the bulk phases that the cytoplasmic membrane separates and the bulk-phase protonmotive force is thermodynamically equal to the degree of membrane energisation. In an alternative model (route B) the energy-coupling proton current is not carried in the bulk aqueous phases and the bulk-phase protonmotive force is of lower energetic magnitude than the degree of membrane energisation

of the bulk-phase transmembrane electrical potential comes from the work of Felle and colleagues [36]. These workers demonstrated that under a variety of conditions the membrane potentials measured with intracellular electrodes in giant cells of *Escherichia coli* correlated well with that measured by the distribution of phosphonium ions, although, regrettably, the effect of protonophores was not tested. McCarthy and coworkers [20] recently summarised the evidence that $BuPh_3P^+$ distribution reflects the bulk-phase membrane potential in bacteria. In this detailed review they could draw attention only to work using spin-labelled phosphonium salts [37] and to resonance Raman spectroscopic evidence obtained with the membrane-permeable quinaldine red cation [38] which indicated that the binding of such salts to energised membranes might cause gross overestimation of the bulk-phase $\Delta\psi$. The criticisms of this method offered by Tedeschi [39] in the case of osmotically-active mitochondria would seem not to be applicable to intact bacterial cells.

Numerous studies, e. g. [40], have shown that phosphonium ions can bind significantly to de-energised bacterial mem-

branes, and it was for this reason that we were careful to base our calculations of values solely on the energy-dependent uptake of $BuPh_3P^+$. Therefore, given the demonstrably rapid permeability of the *Cl. pasteurianum* cell membrane to $BuPh_3P^+$, there is no presently conceivable reason why the distribution of the $BuPh_3P^+$ cation should underestimate the bulk-phase $\Delta\psi$, though we should like to stress that we cannot exclude the possibility that an energy-dependent binding of the ion to the bacterial cytoplasmic membrane may have given a significant overestimate of the calculated bulk-phase transmembrane potential.

Butyricin 7423 as an Inhibitor of the H^+-ATPase of Cl. pasteurianum

At extracellular K^+ concentrations in the 2 – 3 mM range, *Cl. pasteurianum* generated a $\Delta\psi$ value of approximately 100 mV (Fig. 1). That protonmotive hydrolysis of ATP catalysed by its membrane H^+-ATPase was the sole source of this membrane energisation was evidenced by the observation that the efflux to electrochemical equilibrium of both K^+ and $BuPh_3P^+$ could be elicited by either dicyclohexylcarbodiimide or efrapeptin which, at the concentrations employed, are believed to inhibit only the H^+-ATPase. Butyricin 7423 also is capable of inhibiting the H^+-ATPase of *Cl. pasteurianum* in its BF_0 region, both in intact organisms [27] and in reconstituted proteoliposomes [9]. The work described in this paper shows furthermore that butyricin 7423 can mimic the ability exhibited by dicyclohexylcarbodiimide and efrapeptin to dissipate the apparent bulk-phase $\Delta\psi$.

Whilst it might appear that the bactericidal action of butyricin 7423 could adequately be attributed to an inhibition of the hydrolysis of ATP by the membrane H^+-ATPase, there are a number of experimental findings which suggest that such a conclusion is unlikely to prove correct.

(a) Cells of *Cl. pasteurianum* are not killed by normally-lethal concentrations of butyricin 7423 if the minimal glucose medium employed is supplemented with 200 mM K^+ [16], a phenomenon that has also been observed in work with colicin K and *E. coli* [41]. At such high concentrations of K^+ there is no observable bulk-phase $\Delta\psi$ even in the absence of butyricin 7423.

(b) Mutant strain DC3 possesses a H^+-ATPase which has a sensitive to butyricin 7423 of less than 1 % of that displayed by the enzyme complex of its wild-type parent, yet in glucose minimal medium its sensitivity to the bacterial action of butyricin 7423 is 60 – 70 % of that demonstrated by the wild-type organism [16].

(c) Butyricin 7423 at very low concentrations can cause K^+ efflux without a measurable decrease in $\Delta\psi$ (Fig. 4).

(d) With the knowledge that 1 mg of pure butyricin 7423 is equivalent to 100000 AU and that the molecular weight of butyricin 7423 is 32500 it may be calculated that the physiologically active dose of butyricin 7423 is equal to about 1.4 pmol/10^8 cells (10 pmol/mg dry weight of organisms). The inhibitory dose of efrapeptin, a very tight-binding and specific ATPase inhibitor [42], was approximately 1.05 nmol/mg dry weight of *Cl. pasteurianum*. Thus butyricin 7423 is approximately 100-fold more active against *Cl. pasteurianum* than is efrapeptin.

Thus it must be concluded that although butyricin 7423 can undoubtedly inhibit *in vitro* protonmotive ATP hydrolysis by the H^+-ATPase of *Cl. pasteurianum*, analysis of its effects on whole organisms suggests that a second (but non-protonmotive) site of inhibition may be implicated in its ability to disrupt their membrane energy coupling.

Butyricin 7423 Is Not a Simple K^+ Ionophore

That butyricin 7423 is not a simple K^+-ionophore was evidenced by the following observations.

(a) Under conditions in which valinomycin addition could generate a potassium diffusion potential, the addition of appropriate concentrations of butyricin 7423 (e. g. 1 AU per 10^7 organisms) did not give rise to such a diffusion potential (Fig. 4).

(b) Butyricin 7423, although causing a transient K^+ flux [16], could not provoke net K^+ release in *Cl. pasteurianum* mutant strain DC3 whose only known lesion with respect to the sensitive parent strain is that is possesses a H^+-ATPase of diminished sensitivity to inhibition by both butyricin 7423 and dicyclohexylcarbodiimide.

(c) Valinomycin markedly stimulated $^{86}Rb^+/K^+$ exchange in *Cl. pasteurianum* whilst butyricin 7423 inhibited it (Fig. 10).

Some Unresolved Questions

It would appear that the action of butyricin 7423, at its minimum bactericidal concentration, on cells of *Cl. pasteurianum* is not compatible with the formation of a relatively non-discriminating ion-permeable channel in the target cytoplasmic membrane. Furthermore, we believe that the finding that butyricin 7423 inhibited $^{86}Rb^+/K^+$ exchange across the target membrane constitutes compelling evidence that any theory based on its creation of an ion-pore in the membrane can be discounted (see also [7]). Taken together with the evidence that it does not act as a K^+-ionophore or protonophore and can hardly be acting via stoichiometric inhibition of the membrane H^+-ATPase, the molecular mechanism whereby butyricin 7423 dissipates membrane energisation remains to be elucidated.

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