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ON THE PERMEABILITY TO WEAK ACIDS AND BASES OF THE CYTOPLASMIC MEMBRANE OF CLOSTRIDIUM PASTEURIANUM

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SUMMARY

The permeability of the cytoplasmic membrane of Clostridium pasteurianum to weak acids and bases is studied by an osmotic swelling method. It is found that these species cross the cytoplasmic membrane predominantly in their uncharged form. The bioenergetic consequences of this for growing cultures are discussed.

INTRODUCTION

Clostridium pasteurianum is an obligately fermentative anaerobic bacterium which, when grown on simple hexoses, produces large quantities of the weak acids acetate and butyrate, concomitantly lowering the pH of its extracellular medium to values as low as pH 4.0. Typically, 0.6 moles of acetate and 0.7 moles of butyrate are produced per mole of glucose fermented, and it is usually assumed (although direct experimental evidence on this point is apparently lacking) that the excretion of acetate and butyrate is effected by passive diffusion of the uncharged forms of these acids across the cytoplasmic membrane (see e.g. (1)).

It is generally believed that fermentative anaerobes such as Cl. pasteurianum utilise a proton-translocating ATP phosphohydrolase located in the cytoplasmic membrane to create a protonmotive force (alkaline and negative inside) between the cytoplasm and the extracellular space (e.g. 1-5). However, the presence of extracellular acetate plus butyrate in growing cultures would tend to decrease any protonmotivated pH gradient to energetically insignificant values as a consequence of their passage back into the cells. Further, if a pH gradient were to be maintained despite this presence of often high con-

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centrations of acetate and butyrate (25-50 mM is far from uncommon) equilibration of the transmembrane concentration gradients of acetate and butyrate would tend to lead to osmotic swelling of the cells with enhanced leakiness to protons of the cytoplasmic membrane and thence to uncoupling. Indeed, acetic acid is well known to uncouple bacterial free energy conservation at acid pH values (e.g. 6). As part of a systematic study of the bioenergetics of Cl. pasteurianum we have therefore sought to obtain experimental data on the permeability of the cytoplasmic membrane of this organism to the charged and uncharged forms of the weak acid acetate and the weak base ammonia, and we here report evidence that, in common with their passage across other biological membranes, these species cross the membrane of this organism in the uncharged form. The energetic consequences of this phenomenon for the growing organism are discussed.

MATERIALS AND METHODS

Clostridium pasteurianum MR505, a granulose-negative mutant derived from strain ATCC 6013 (7), was grown in glucose-limited chemostat culture (dilution rate 0.2) using a defined medium consisting of (per litre): 2.8 g glucose, 0.66 g NH_4Cl, 0.123 g $MgSO_4$, 0.596 g KCl, 0.069 g Fe-Na EDTA, 4 mg p-amino benzoic acid, 0.24 mg biotin, 50 mmol sodium phosphate pH 7.0 and 0.5 g cysteine HCl. The steady-state extracellular pH value in this culture was pH 6.1. Protoplasts were prepared by suspending organisms harvested from approximately 200 ml of culture in 50 ml of 25 mM potassium phosphate buffer pH 7.0 containing 6mM $MgSO_4$, 0.46M lactose and 10 mg hen egg white lysozyme, and incubating this suspension for approximately 30 minutes at 37°C, essentially as described (4), until formation of protoplasts was complete as judged by microscopic examination. The protoplasts were sedimented by centrifugation at 10,000 x g for 5 minutes, resuspended in 5 ml of the same buffer mixture lacking lysozyme and kept on ice. Protoplasts so prepared were stable for at least 6 hours as judged by their absorbance when 0.05 - 0.1 ml samples were diluted into a solution of 0.5M lactose.

Osmotic swelling experiments were performed in cuvettes (10 mm light path) by the addition of small (<0.1 ml) volumes of the stock protoplast suspension to 3 ml of a 0.5M solution of the appropriate salt, with subsequent additions of <0.01 ml of ethanolic ionophore solutions being made as detailed in the legends to the Figures. The absorbance at 600 nm of each suspension was continuously monitored using a Pye-Unicam SP1800 spectrophotometer.

Chemicals were obtained from previously-described sources (4,8).

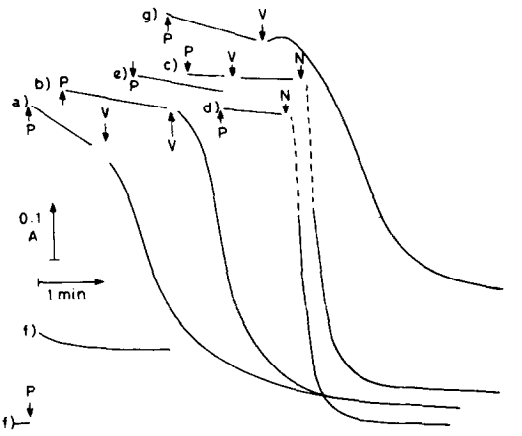

Fig. 1. Osmotic swelling of protoplasts of Cl. pasteurianum MR505 in certain salt solutions. 80 µl of a stock suspension of protoplasts (5 mg protein/ml) were added at the points marked (P) to 3 ml of a 0.5M solution of one of the following salts: (a) KSCN, (b) KNO_3, (c) CH_3COOK (d) CH_3COOK, (e) $NaNO_3$, (f) CH_3COONH_4, (g) NH_4NO_3, such that the optical density at 600 nm extrapolated to the time of addition was 0.70. Valinomycin (V) and Nigericin (N) were added as indicated, each to a final concentration of 2 µg/ml. In each case the final optical density attained was 0.12, although the traces have been displaced for reasons of clarity.

RESULTS

As is well known, a convenient way to examine the permeability properties of vesicular systems is to study their osmotic behaviour in various solutes, as monitored by light-scattering changes. This method has been used for example with protoplasts of Micrococcus lysodeikticus (9) and of other bacteria (10), and with mitochondria (11), chloroplast thylakoids (12) and rod outer segment disc membranes (13). Figure 1 shows the changes in absorbance that are seen when 80µl of a suspension of protoplasts of Cl. pasteurianum MR505 was added to 3 ml of 0.5M KSCN (trace (a)) or 0.5M KNO_3 (trace (b)); it may be seen that the SCN^- salt caused a much faster decrease in light scattering by the protoplasts than did an equiosmolar concentration of the NO_3^- salt; this we ascribe to the severe chaotropic action of SCN^- at these concentrations (14). The addition of valinomycin to these suspensions caused a relatively rapid decrease in light scattering, consistent with the widely-held view that nitrate and thiocyanate salts can rapidly permeate biological membranes in

their charged forms (see e.g. (15)) and that the passage of a counterion, K^+ facilitated by the presence of valinomycin, leads to a rapid osmotic swelling (e.g. (10,11)). The somewhat slower rate of swelling induced by valinomycin when 0.5M SCN^- was present is presumably due to a space-charge effect of SCN^- ions bound to the membrane (14,16).

When the protoplasts were instead resuspended in 0.5M potassium acetate their rate of swelling, which in the absence of ionophores was extremely slow, was not increased by the presence of valinomycin (trace (c)) but was enormously increased by the addition of the ionophore nigericin, either subsequently (trace (c)) or alone (trace (d)). Similar rates of swelling to those observed in 0.5M potassium acetate were observed when the protoplasts were resuspended in 0.5M sodium acetate, either in the presence or absence of ionophores (data not shown). Since nigericin catalyses an electroneutral antiport of K^+ and H^+ (e.g. (10,17)) these findings may be taken to indicate that, even at pH 7.0, the protoplast membranes have a negligible permeability to the charged form of acetate (i.e. CH_3COO^-) and a very substantial permeability to acetate in its uncharged form (i.e. CH_3COOH).

The final traces of Fig 1 show the light-scattering changes observed when the same quantities of protoplasts were suspended in 0.5M solutions of sodium nitrate (trace (e)), or ammonium acetate (trace (f)) or ammonium nitrate (trace (g)). The rate of swelling of the protoplasts in $NaNO_3$ was similar to that observed in KNO_3, and suggests that a weak chaotropic effect of NO_3^- causes a relatively non-selective permeabilisation of the cytoplasmic membrane of these protoplasts. The rate of protoplast swelling in ammonium acetate, however, (trace (f)), was too fast to measure when the contents of the spectrophotometer cuvette were mixed manually and shows beyond reasonable doubt that the membranes of these protoplasts are exceedingly permeable to ammonia in its uncharged form. Finally, the slow rate of protoplast swelling observed in 0.5M NH_4NO_3 shows that the protoplast membrane was not significantly permeable to the charged ammonium ion. However, when the permeability of the membrane to NH_4^+ was

enhanced by the addition of valinomycin then, as expected, osmotic swelling of the protoplast took place. The lag before the initiation of such swelling can be ascribed to release of intraprotoplast K^+, as it was not observed in KNO_3 (trace (b)), whilst the fact that the valinomycin-induced rate of swelling in NH_4NO_3 was noticeably less than that in KNO_3 indicates that, even when the permeability of the protoplast membrane to NH_4^+ is made artifically high by the presence of valinomycin, a kinetically significant efflux of uncharged NH_3 can slow down the rate of swelling, a point elaborated upon more fully in the discussion.

DISCUSSION

Although it is well known that acetic acid can indeed cross biological membranes solely in the uncharged form, very little consideration has been given to the energetic consequences of this fact for those fermentative bacteria which excrete large quantities of acetate and other weak fatty acids into their culture media. The concentrations of acetate and butyrate (charged plus uncharged) in our chemostat culture in its steady state were each approximately 7mM (G. Rodger, unpublished observation). Obviously, in a batch culture initially containing excess glucose these concentrations can reach exceptionally high levels and, in concert with the concomitant lowering of the environmental pH, may be the prime cause of the cessation of growth of such cultures. In complex anaerobic consortia such as those implicated in methanogenesis, unbalanced acetogenesis can be the prime cause of digester failure (18). Even at pH 6.5, concentrations of acetate as low as 10mM were sufficient to inhibit almost completely oxalate uptake into intact cells of Pseudomonas oxalaticus (19), a process believed to be driven solely by the transmembrane pH gradient in this organism. It may therefore seem surprising that Cl. pasteurianum might be thought to maintain a transmembrane pH gradient of any energetic significance in normally growing cultures. Indeed it has been found that the internal pH of Cl. pasteurianum does not remain constant but falls progressively as the pH of its external medium

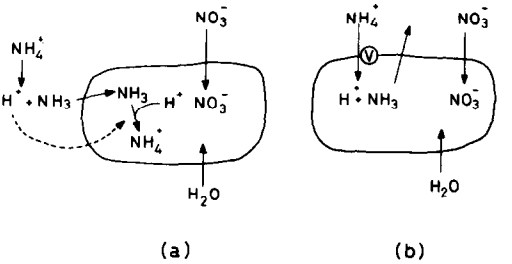

Fig. 2. Diagrammatic representation of the mechanism of swelling of Cl. pasteurianum protoplasts in ammonium nitrate in the absence (a) or presence (b) of valinomycin. For further explanation, see text.

becomes more acidic during its growth in batch culture (2). Even so, it was reported that equilibration of internal and external pH values was never achieved, a ΔpH (internal alkaline) of 0.4-0.8 pH units being sustained throughout growth (2). We can only suggest that several factors including the use of the dextran-impermeable space for correcting observed isotope distributions, the use of excessive concentrations of uncoupler and ATPase inhibitors and the binding or metabolism of the probes used by these authors may have conspired together to cause an overestimation of ΔpH. It is noteworthy that these authors found that the pH gradient estimated by their method was in some cases almost completely insensitive to 0.2mM carbonyl cyanide m-chlorophenylhydrazone and that discrepant estimates of the pH gradients were obtained when different probes were used.

The slightly faster rate of swelling observed in NH_4NO_3 relative to that observed in KNO_3 in the absence of ionophores deserves some comment, particularly in view of the claim (20) that the cytoplasmic membrane of Cl. pasteurianum is impermeable to NH_3 but permeable to the charged NH_4^+ species. A model of the swelling behaviour in NH_4NO_3 is diagrammed in Fig 2. In the absence of valinomycin (Fig 2(a)), nitrate ion and uncharged ammonia cross the membrane; protonation of NH_3 by intracellular acid-base groups leads to osmotic swelling, leakiness of the protoplast membrane to protons and subsequent uptake of protons to neutralise the pH gradient and membrane potent-

ial formed if the diffusive fluxes remained uncompensated. In the presence of valinomycin NH_4^+ may be rapidly taken up, but a kinetically significant efflux of uncharged NH_3, together with a significant buffering of the protons left in the internal phase, results in a slowing (relative to that of K^+ in the presence of valinomycin) of the influx of osmotically active positive charges to balance the nitrate ions passing down their concentration gradient. We further note that the protonmotivated uptake of NH_4^+ intimated by Kleiner and Fitzke (20) would, given the high permeability of these membranes to NH_3 demonstrated here, lead simply to uncoupling and dissipation of metabolic energy. Whilst the presence of a steady-state transmembrane pH gradient (alkaline inside) would indeed tend to exacerbate the (solely kinetic) problem of growth at low pH under conditions of ammonium limitation, as these authors (20) and others (21) have pointed out, the fact that we have been able to observe such a high native permeability of the cytoplasmic membrane of Cl. pasteurianum to acetic acid and ammonia would suggest that this organism solves this problem by generating only an energetically insignificant transmembrane pH gradient. Finally, it is expedient to warn against the use (20) of 2,4-dinitrophenol as an uncoupler in suspensions of Clostridium species, for butyric clostridia can actively reduce this molecule (22); the path to understanding the bioenergetic consequences of this activity remains untrodden.

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