Journal of General Microbiology (1987), **133**, 259–266. *Printed in Great Britain*

The Roles of Osmotic Stress and Water Activity in the Inhibition of the Growth, Glycolysis and Glucose Phosphotransferase System of *Clostridium pasteurianum*

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(*Received 25 April 1986; revised 18 August 1986*)

Growth of *Clostridium pasteurianum* was inhibited in media of high solute content. At equal osmolarities, 'permeant' solutes (glycerol and acetamide) were much less growth-inhibitory than 'non-permeant' solutes (KCl and xylitol). Glycolysis by washed cell suspensions was inhibited by these solutes in parallel with growth. However, in their inhibition of glucose 6-phosphate dissimilation by permeabilized cells the distinction between 'permeant' and 'impermeant' solutes was significantly less marked. The glucose phosphotransferase system (PTS) of intact cells was much more strongly inhibited by 'non-permeant' than by 'permeant' solutes. It was concluded that the predominant inhibitory effects on this organism of media of high solute content are due not to the low water activity of such media *per se*, but to the creation of an osmotic pressure across the bacterial cytoplasmic membrane, which acts to inhibit the glucose PTS by which the organism effects glucose uptake. Parallel measurements of the effects of xylitol on both glycolysis and the activity of the glucose PTS suggested that despite this correlation between the osmotic inhibition of growth, glycolysis and the PTS, the flux-control coefficient of the PTS on glycolysis did not exceed 0·2 under the conditions used.

INTRODUCTION

The growth of many non-halophilic bacteria is inhibited in media of high osmolarity, a fact which is usually ascribed to the low water activity (a_w) of such media (Scott, 1957; Corry, 1973; Brown, 1976; 1978; Gould & Measures, 1977; Kushner, 1979; Jones & Greenfield, 1986), and which is widely exploited in food preservation (Scott, 1957; Troller, 1973; Duckworth, 1975; Davies *et al.*, 1976). In many cases, the targets of these measures are anaerobic bacteria (Roberts & Smart, 1976). Yet, given the high permeability to water, relative to that of other solutes, of biological membranes (Fettiplace & Haydon, 1980), the predominant mechanism of the non-specific inhibition of growth by added solutes might lie either in the inhibitory properties of a low a_w to bacterial growth and metabolism *per se* or to the creation, by the extracellular solutes, of an inhibitory (osmotic) pressure drop across the semi-permeable bacterial cytoplasmic membrane. Motivated also by the knowledge that the benefits of anaerobic micro-organisms in biotechnological processes (Morris, 1982) are most likely to be realized in media which can support a high biomass concentration, and which may therefore have a low a_w (Esener *et al.*, 1981), we have sought to determine the basis for the inhibition of bacterial growth in media of high solute content, using the obligately anaerobic, saccharolytic, Gram-positive bacterium *Clostridium pasteurianum*. A preliminary account of this work has been given elsewhere (Walter & Kell, 1986).

Abbreviations: α-MG, methyl α-D-glucopyranoside; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

METHODS

Strains, Clostridium pasteurianum ATCC 6013, and its mutant derivative 6013-ES1, which lacks granulose phosphorylase and cannot therefore mobilize its endogenous reserves (Mackey & Morris, 1974), were maintained as described by Clarke *et al.* (1982). The organisms were grown in 2% (w/v) glucose minimal medium at 37 °C (Clarke *et al.*, 1982), and growth was assessed by measuring the optical density at 680 nm in a Bausch and Lomb Spectronic 70 spectrophotometer, converting where necessary to values of dry wt or cell number via previously obtained calibration curves (Clarke *et al.*, 1982).

Protoplasts. These were obtained by lysozyme treatment, and their osmotic swelling behaviour was assessed turbidimetrically, as described by Kell *et al.* (1981).

Glycolysis by washed cell suspensions. This was assessed as the rate of acid production (Kell & Walter, 1986), since acetic and butyric acids are by far the major fermentation end-products of this organism (Thauer *et al.*, 1977), at least in the medium described (data not shown). Cells were harvested, washed and resuspended in a basal medium consisting of 25 mM-triethanolamine/H_3PO_4, pH 6·5, containing 50 mM-KCl, 5 mM-$MgSO_4$ and 0·05% (w/v) cysteine.HCl. Glucose was added to a concentration of 20 mM. All procedures were done anaerobically, and pH changes were recorded in a vessel (Hitchens & Kell, 1982), and using the apparatus (Hitchens & Kell, 1984), described previously. The reaction volume was 8 ml and the temperature was 37 °C. Other media and additions are given in the legends to Figures.

Sugar uptake. This was assessed in the same medium as that used for the measurement of glycolysis but using [U-^{14}C]glucose or methyl (α-D-[U-^{14}C]gluco)pyranoside ([^{14}C]αMG), a non-metabolizable analogue of glucose which serves as a substrate for the glucose phosphotransferase system (PTS) of this organism (Booth & Morris, 1982; Mitchell & Booth, 1984). Samples (0·5 ml) were taken, filtered using Whatman GF/C glass fibre filters and washed using 2 × 5 ml of basal medium. The volume of the reaction mixture was 5 ml and 0·3 μmol of radioisotope [1 μCi (37 kBq)] was present. Concentrations of sugars, sugar analogues and carbon sources are given in the legends to the Figures.

Miscellaneous assays. Fermentation end-products were measured by GLC (Gottschal & Morris, 1981). ATP was measured in a Packard Luminometer using the firefly luciferase assay and a protocol supplied by the manufacturers, whilst glycolytic intermediates were measured enzymically according to standard procedures (Bergmeyer, 1974).

Chemicals and biochemicals. These were obtained from Sigma or BDH, and were of the highest grade available; methyl(α-D-[U-^{14}C]gluco)pyranoside was obtained from Amersham. Water was singly distilled in an all-glass apparatus.

RESULTS AND DISCUSSION

The growth rate of *C. pasteurianum* 6031-ES1 in batch cultures decreased as the total osmolarity of the medium increased (Fig. 1). The osmolarity of the glucose minimal medium at the start of growth was 275 mosM. It should be noted that osmolarity rather than osmolality was used to describe solute concentrations for two reasons. Firstly, the major purpose was to compare permeant and impermeant solutes, and secondly because tabulated data were not available to describe the non-ideal behaviour of all the solutes selected. Other experiments (data not shown) indicated that the effects of these solutes were bacteriostatic rather than bactericidal and that growth inhibition was not relieved by inclusion in the growth medium of the 'compatible solutes' (Brown, 1976) proline, glutamate, betaine, sarcosine or choline (0·5 mM). Glycerol and acetamide, at the same osmolarity (and hence approximately equal osmolality) were significantly less inhibitory to growth than were KCl and xylitol. Other workers have also noted effects of this nature, without giving a mechanistic explanation for them (Spiegelberg, 1944; Baird-Parker & Freame, 1967; Kang *et al.*, 1969; Strong *et al.*, 1970; Marshall *et al.*, 1971; Jakobsen & Trolle, 1979). However, glycerol and acetamide are small, neutral molecules, and it may be expected that they might penetrate the bacterial membrane relatively rapidly, whilst the ionic KCl and the sugar alcohol xylitol might be expected to be rather impermeant species under conditions *in vivo* [where the internal KCl concentration of these Gram-positive organisms is approximately 0·4 M (Clarke *et al.*, 1982)]. Since the enclosed volume fraction of a microbial culture containing 1–2 mg dry wt organisms ml^{-1} is only about 0·002–0·004 (Harris & Kell, 1985), it may be predicted that whilst equal osmolarities of 'permeant' and 'impermeant' solutes will have equal effects on the homogeneous a_w of the system (given the high permeability of biological membranes to water), 'impermeant' but not 'permeant' solutes may additionally affect the osmotic pressure across the bacterial cytoplasmic membrane (Morris, 1974). We therefore chose to pursue this line of reasoning.

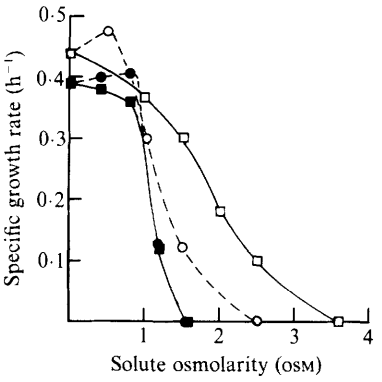

Fig. 1

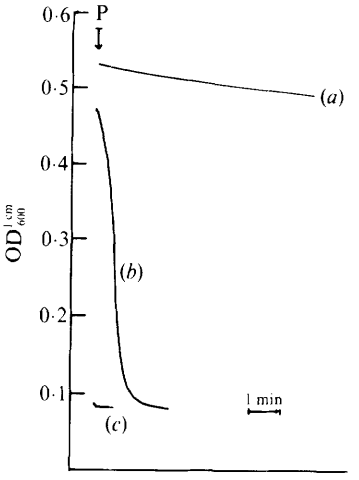

Fig. 2

Fig. 1. Effect of added solutes on the growth of *C. pasteurianum* 6013-ES1 in batch culture. Bacteria were grown in a glucose minimal salts medium. The solutes added were xylitol (■), glycerol (□), KCl (●) and acetamide (○). Growth was monitored by assessing the increase in optical density at 680 nm.

Fig. 2. Osmotic behaviour of protoplasts of *C. pasteurianum* 6013-ES1. Protoplasts were resuspended at an optical density of approximately 60 in 25 mM-potassium phosphate buffer, pH 7·0, containing 6 mM-$MgSO_4$ and 0·46 M-lactose. At the point marked P, 35 µl of the suspension were added to 4 ml of a 1 osM solution of the solute of interest and the optical density at 600 nm was followed. The solutes tested were xylitol or KCl (*a*), glycerol (*b*) and acetamide (*c*).

Light-scattering methods provide an extremely convenient means by which to assess the osmotic behaviour and solute permeability of biological membranes (Henderson, 1971; Uhl *et al.*, 1980; Kell *et al.*, 1981). The swelling behaviour of bacterial protoplasts suspended in media containing high concentrations of the test solutes used in Fig. 1 was therefore studied spectrophotometrically (Fig. 2). Protoplasts were stable in xylitol and KCl, but glycerol permeated them on a rapid time-scale relative to that of growth. The permeability of the cell membrane to acetamide was too great to be measured using manual mixing. Thus, there was a clear inverse correlation, using structurally unrelated substances, between the ability of a solute to cross the clostridial cytoplasmic membrane and its growth-inhibitory properties at high concentrations.

Since, when all nutrients are in excess, it is thought that growth in batch culture is limited more by catabolism (i.e. by the rate of provision of energy as ATP) than by anabolism (Ingraham *et al.*, 1983), the previous results would suggest that glycolysis, the only source of ATP production in this organism, should be affected by the external osmotic conditions in a manner similar to their effects on growth. The effects of the above solutes on the glycolytic activity of washed cell suspensions were therefore examined.

Strain 6013-ES1 was used in the study of the effect of solutes on glycolytic rate (Fig. 3) since it is unable to degrade its intracellular storage polymer granulose (Mackey & Morris, 1974). Thus, all the glycolytic acid production observed was derived from exogenous glucose. The inhibitory effect of the various solutes on growth (Fig. 1) was qualitatively similar to their inhibitory effects on glycolysis (Fig. 3), consistent with the view that reactions connected with the production of ATP are indeed amongst those most rate-determining to growth in batch cultures. The finding that the distinction between the 'permeant' and 'impermeant' solutes was even more marked in washed cell suspensions than in growing cells may be accounted for by the relative time-scales of the experiments. Even the 'impermeant' solutes have a finite permeability (Fig. 2), which may be expected to be more important to the present distinction the longer the duration of the

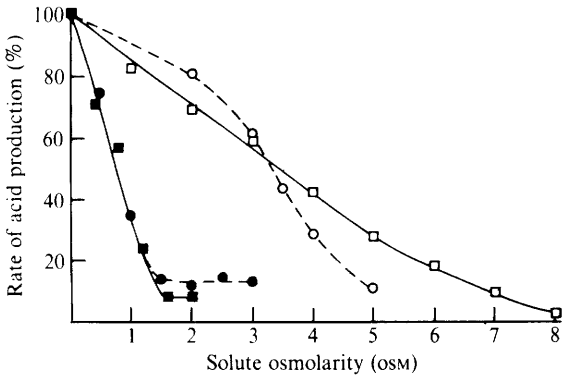

Fig. 3

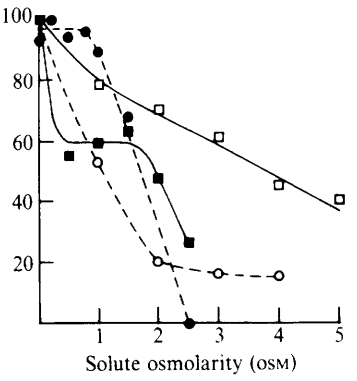

Fig. 4

Fig. 3. Effect of added solutes on the rate of glycolytic acid production by intact cells of *C. pasteurianum* 6013-ES1. The reaction medium contained, in a volume of 8 ml, 25 mM-triethanolamine/H_3PO_4, pH 6·5, 50 mM-KCl, 5 mM-$MgSO_4$, 0·05% (w/v) cysteine.HCl and 20 mg dry wt cells, together with solutes of the osmolarities indicated. The temperature was 37 °C and glycolysis was initiated by the addition of 20 mM-glucose. The solutes used were xylitol (■), glycerol (□), KCl (●) and acetamide (○). 100% corresponds to a rate of acid production of 0·250 μg-ion H^+ min^{-1} (mg dry wt)$^{-1}$.

Fig. 4. Effect of added solutes on the rate of glycolytic acid production by toluenized cells of *C. pasteurianum* 6013-ES1. Washed cells (20 mg dry wt) of *C. pasteurianum* were suspended in 8 ml of the medium described in the legend to Fig. 3. Toluene/ethanol (0·1 ml of 1 : 10, v/v) was added to this suspension, followed by NAD^+, ADP and ATP to final concentrations of 1, 0·8 and 2 mM respectively. Acid production was initiated by the addition of glucose 6-phosphate (K^+ salt) to a final concentration of 5 mM. The temperature was 37 °C and 100% corresponds to a rate of acid production of 0·075 μg-ion H^+ min^{-1} (mg dry wt)$^{-1}$.

experiment, and the growth experiments were considerably more protracted than were those in which acid production by washed cells was monitored.

Given the absence of any obvious structural relationships between the compounds chosen, the strong dependence of the ability of these compounds to inhibit glycolysis on whether or not they could rapidly cross the bacterial membrane suggested that their site of inhibitory action must be located in the cytoplasmic membrane itself. At least two predictions follow from this analysis. Firstly, there should be a much less marked difference in the inhibitory effect of 'permeant' and 'impermeant' solutes on the glycolytic activities in cells from which the osmotic barrier has been removed. Secondly, the activity of the membrane-located glucose uptake system of this organism itself should be more sensitive to the 'impermeant' than to the 'permeant' solutes. In this regard, it should be mentioned that Roth *et al.* (1985*a*, *b*) have shown directly that high osmotic pressures can indeed inhibit several sugar transport systems in *Escherichia coli*. We therefore measured these activities in *C. pasteurianum*.

As with many other cell types (Felix, 1982), toluene destroys the integrity of the cytoplasmic membrane of *C. pasteurianum* (Kell & Walter, 1986). Since this organism lacks hexokinase, and a phosphoenolpyruvate (PEP)-dependent PTS is thought to represent the sole means of glucose uptake (Booth & Morris, 1982; Mitchell & Booth, 1984), glycolysis was completely inhibited by treatment of cells (5–20 mg ml^{-1}) with toluene [1%, v/v, of toluene/ethanol (1 : 10, v/v) (Kell & Walter, 1986)]. However, as with dextran-sulphate-permeabilized mouse L-cells (Clegg, 1984), acid production from glucose 6-phosphate could be restored by the addition of NAD^+ and ATP (Kell & Walter, 1986). In this case, following a short lag, several acidic products were formed, including 2- and 3-phosphoglyceric acid. Glycerol still appeared to be somewhat less inhibitory (on an osmolar basis) to acid production than were the other solutes, but the distinction between acetamide and the 'impermeant' xylitol and KCl was indeed largely abolished (Fig. 4). That all compounds tested still inhibited glycolytic acid production in toluenized bacteria to some extent

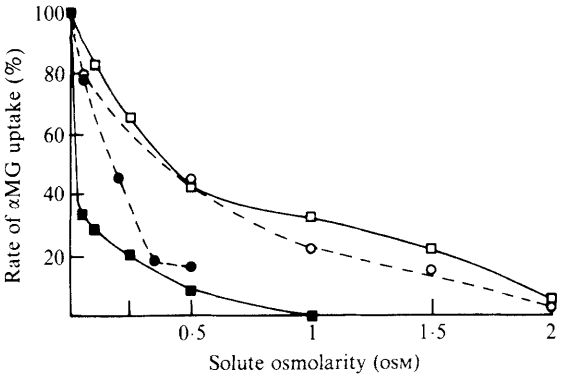

Fig. 5

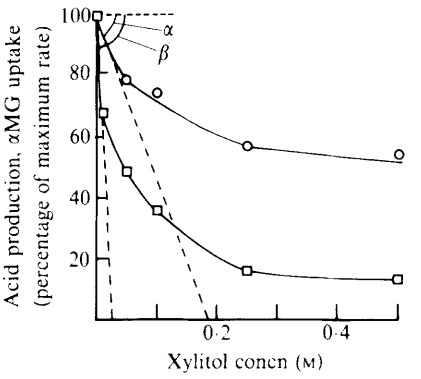

Fig. 6

Fig. 5. Effect of added solutes on the rate of uptake of [^{14}C]αMG via the glucose PTS of *C. pasteurianum* 6013. No exogenous glucose was present and the reaction was initiated by the addition of αMG [1 μCi μmol^{-1} (37 kBq μmol^{-1})] to a final concentration of 60 μM. The following solutes were also present at the osmolarities indicated: xylitol (■), glycerol (□), KCl (●) and acetamide (○). 100% corresponds to a rate of uptake of αMG of 150 pmol min^{-1} (mg dry wt)$^{-1}$.

Fig. 6. Estimation of the flux-control coefficient of the glucose PTS for glycolysis by washed cells of *C. pasteurianum* 6013-ES1. Glycolytic acid production (○) and the uptake of [14C]αMG (□) were determined in parallel, and as described in the legends to Figs 3 and 5, except that glucose was present at a concentration of 600 μM. αMG was added 30 s after glycolysis had been initiated, at which time a steady rate of acid production had been obtained. The angles α and β have the values 70° and 87°, and the ratio of their tangents, which equals the flux-control coefficient, in this case equals 0·14. In no case was a value exceeding 0·2 determined in replicate experiments.

shows that there are direct effects of these compounds on glycolytic enzymes additional to those on the glucose PTS and their ability to lower the a_w of the system.

To assess the effects of these solutes on the activity of the PTS itself, we exploited the substrate αMG, a non-metabolizable analogue of glucose which is nonetheless taken up by the PTS (Booth & Morris, 1982). For this experiment the endogenous metabolism of granulose in strain 6013 provided the PEP substrate. All of the solutes tested inhibited the activity of the glucose PTS but the 'impermeant' xylitol and KCl were much more inhibitory than were the 'permeant' glycerol and acetamide (Fig. 5). As with the measurement of acid production by washed cell suspensions, and for the reasons discussed above, the distinction was more marked than when growth was the variable considered.

Thus far, we have adduced evidence that the glucose PTS might constitute the 'primary' locus at which might be exerted the inhibitory effects of media of high solute content on the growth of this organism. However, it is apparent that the PTS (Fig. 5) was sensitive to lower concentrations of solutes than were growth (Fig. 1) or glycolysis (Fig. 3). This illustrates a facet of the metabolic control theory of Kacser & Burns (1973) and Heinrich & Rapoport (1974), the implications of which have been discussed more recently by Groen *et al.* (1982), Westerhoff *et al.* (1984) and Kell & Westerhoff (1986*a*, *b*). Fluxes are rarely (if ever) completely controlled by individual enzyme reactions. Thus, it would be inappropriate to claim, for instance, that the PTS is 'the' site of inhibition of the growth of *C. pasteurianum* by, say, xylitol. Furthermore, while the finding that 'impermeant' solutes are more inhibitory to growth and glycolysis than are 'permeant' ones remains valid, the differing sensitivities of glycolysis and the PTS to solute concentrations suggested that the PTS might have a low flux-control value in the glycolytic pathway.

The flux-control coefficient provides a quantitative (and dimensionless) representation of the extent to which a particular enzyme controls a particular flux (Westerhoff *et al.*, 1984; Kell & Westerhoff, 1986*a*, *b*), and is defined as the fractional change in pathway flux divided by the

fractional change in the activity of the enzyme of interest. One means by which enzyme activities may be varied is by the use of inhibitors, and the fact that xylitol is an inhibitor of the PTS (Fig. 5) allowed us to use it to estimate the flux-control coefficient of the PTS on glycolysis. For a specific inhibitor (Groen *et al.*, 1982; Kell & Westerhoff, 1986*a*, *b*), the flux-control coefficient of an enzyme is given by the ratio of the initial slope of a normalized plot of the flux versus the inhibitor concentration to that of a normalized plot of the enzyme turnover number versus the inhibitor concentration. The present approach is particularly useful in the case of the first enzyme in a pathway, since the concentration of its substrate is held fixed by the experimenter, a necessary condition for this general type of measurement. If, for a given pathway, the inhibitor is not specific to a particular target enzyme of interest, the flux-control coefficient so estimated represents a maximum value. It should be noted that such estimations must be made under identical conditions and that the value for the flux-control coefficient pertains only to those conditions.

In order to determine the flux-control coefficient, it was necessary to obtain values for both PTS and glycolytic activities in the same experiment (Fig. 6). In this case, cells of strain 6013-ES1 were used and exogenous glucose was present at a concentration of 600 μM in addition to the 60 μM-[U-^{14}C]αMG which served as a 'probe' of the turnover number of the PTS. This concentration of glucose was chosen so that significant αMG uptake could be observed (since glucose is a competitive inhibitor of αMG uptake) whilst permitting a steady rate of glycolytic acid production to be maintained throughout the duration of the experiment. Control experiments (not shown) indicated that the present concentration of αMG did not have a measurable effect upon the glycolytic rate. The relevant slopes for the calculation of the flux-control coefficient are equal to the tangents of the angles α and β in Fig. 6, which gave a value of 0·14 for the flux-control coefficient. This type of experiment thus illustrates the fact that the extent to which a 'target' enzyme is inhibited by a particular treatment may bear only a rather loose or indirect relationship to the effect of the inhibitor on the flux through the metabolic pathway of which that enzyme is a component.

We conclude that in *C. pasteurianum* it is the osmotic pressure induced across the bacterial cytoplasmic membrane, and not simply the a_w of the medium *per se*, which constitutes the more significantly growth-inhibitory physical property of media of high solute content. The biophysical basis for this inhibition, presumably directed at the membrane-located enzyme II portion of the glucose PTS, remains unknown. However, it seems most plausible to suggest, as have others (Macdonald, 1984; Roth *et al.*, 1985*b*), that a pressure-induced change in the conformation of the membrane lipids and/or proteins underlies the observed loss of catalytic activity displayed by the glucose PTS.

We thank the Biotechnology Directorate of the Science and Engineering Research Council, UK, for a studentship to R.P.W. and for generous financial support.

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