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Effects of pCO_2 on the growth and metabolism of *Clostridium sporogenes* NCIB 8053 in defined media

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The effects of the partial pressure of carbon dioxide (pCO_2) on the growth rate of *Clostridium sporogenes* NCIB 8053 in batch culture were investigated in defined minimal media. Depending upon the growth medium, CO_2 was stimulatory or inhibitory to growth. The absolute CO_2 requirement for growth displayed by *Cl. sporogenes* in certain media was shown to be due to the involvement of CO_2 in the synthesis of branched-chain amino acids. High concentrations of CO_2 were growth inhibitory except in complex media. In media where an optimal pCO_2 was observed it was approximately 0·45 atm. The pattern of fermentation end-products displayed by this organism was also modified by the pCO_2. The equilibria of the different species of 'CO_2' present in microbiological media are described. Finally, the metabolic control theory of Kacser, Burns, Heinrich and Rapoport has been applied to the results obtained, to provide a quantitative description of the effects of pCO_2 on the growth rate of *Cl. sporogenes*.

Carbon dioxide has been shown to inhibit the growth of numerous micro-organisms (e.g. Coyne 1933; Gladstone *et al.* 1935; Chen & Glutmanis 1976; Jones & Greenfield 1982; Eklund 1984; Teixeira de Mattos *et al.* 1984), even though they may display a requirement for it (Rockwell & Highberger 1927; Charles & Roberts 1968; Dehority 1971; Kritzman *et al.* 1977; Jones & Greenfield 1982). Indeed, the antimicrobial action of CO_2 atmospheres has been exploited in a variety of systems for the preservation of foods (Smith 1963; Sutherland *et al.* 1977; Gill & Tan 1980; Blickstad *et al.* 1981). At a metabolic level the primary sites at which CO_2 exerts its effects may be presumed to be associated with enzymatic carboxylation and decarboxylation reactions (Wood & Stjernholm 1962; Wood & Utter 1965; Wimpenny 1969; Jones & Greenfield 1982). In addition, CO_2 may act as an inducer or repressor of enzyme synthesis (Jones & Greenfield 1982; Bowien & Leadbeater 1984). Nonetheless a clear mechanistic understanding of the effects of CO_2 on the growth and metabolism of any given micro-organism is not yet available.

In a recent series of studies we have developed a defined minimal medium for the growth of *Clostridium sporogenes* NCIB 8053 (Lovitt *et al.* 1987b) and have described the growth energetics of this organism in both batch and chemostat cultures (Lovitt *et al.* 1987a). This organism is of particular interest since it may derive its free energy (1) by the fermentative dissimilation of glucose (Princewill 1978; Lovitt *et al.* 1987a, 1987b); (2) by means of Stickland reactions using pairs of amino acids (Stickland 1934, 1935a, 1935b; Seto 1980; Lovitt *et al.* 1987a); or (3) by a 'mixed Stickland' reaction in which glucose serves as exogenous electron donor and an amino acid such as proline or phenylalanine as terminal electron acceptor (Lovitt *et al.* 1986, 1987a, 1987b). We have also shown (Lovitt *et al.* 1986) that the reduction of D- or L-proline by cells grown in its presence is

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coupled to the vectorial transmembrane ejection of protons.

During these studies no special attempts were made to optimize the pCO_2 under which the organisms were grown, although it was noted that that usually chosen ($pCO_2 = 1$ atm) was somewhat suboptimal for growth. Therefore, and in view of an early report (Gladstone *et al.* 1935) that *Cl. sporogenes* requires CO_2 for growth, a systematic study of the effects of CO_2 on this organism was undertaken.

Materials and Methods

ORGANISM

Clostridium sporogenes NCIB 8053 was used throughout and was maintained as previously described (Lovitt *et al.* 1987b).

MEDIA

These were based on the low phosphate basal medium (LPBM) described by Lovitt *et al.* (1987b), of the following composition (g/l unless stated): K_2HPO_4, 2·1; KH_2PO_4, 0·3; $MgCl_2.6H_2O$, 0·2; NH_4Cl, 5·0; 0·01% w/v resazurin solution, 1 ml; trace element solution, 10 ml; vitamin solution, added after sterilization, 10 ml. The trace element solution contained (g/l): nitrilotriacetic acid, 12·8; $FeSO_4.7H_2O$, 0·1; $MnCl_2.4H_2O$, 0·1; $CoCl_2.2H_2O$, 0·17; $CaCl_2.2H_2O$, 0·1; $ZnCl_2$, 0·1; $CuCl_2$, 0·1; H_3BO_4, 0·01; $NaMoO_4.2H_2O$, 0·01; NaCl, 1·0; $NaSeO_3$, 0·017; $NiSO_4.6H_2O$, 0·026; $NaWO_4.2H_2O$, 0·1. The vitamin solution contained (mg/l): thiamine HCl, 50; biotin, 5; *p*-aminobenzoic acid, 5; nicotinic acid, 500.

Amino acid complete medium (MACC) (Lovitt *et al.* 1987b) was LPBM supplemented with the following L-amino acids at the following final concentrations (mmol/l): glycine, 3; valine, 2; isoleucine, 1; arginine, 2; leucine, 1; histidine, 1; methionine, 1; phenylalanine, 1; tryptophan, 1; tyrosine, 0·125. Essential amino acid/fatty acid (EAA/FA) medium was the same as MACC medium except that L-leucine, L-isoleucine and L-valine were replaced by 1 mmol/l each of 2-methyl propionic acid, 3-methyl butyric acid and 2-methyl butyric acid. Tryptone medium was MACC medium supplemented with 1% w/v tryptone. Each of the above media contained (final concentrations in mmol/l): L-proline, 20; glucose, 10. Valine/proline medium was EAA/FA medium without glucose but containing (final concentrations in mmol/l): acetate, 10; L-valine, 30; L-proline, 60. Malate medium was EAA/FA medium without glucose and containing (final concentrations in mmol/l): sodium DL-malate, 30; L-proline, 60. In each case the above media were pre-reduced by the addition of 3 mmol/l of L-cysteine before inoculation.

CULTIVATION OF ORGANISMS

Inocula were grown overnight in pressure tubes (Bellco, Vineland, NJ, USA) as described by Lovitt *et al.* (1987b). Ten ml of an overnight culture were then injected into a fermentation vessel of 450 ml working volume (LH Engineering, Stoke Poges, Bucks, UK), containing pre-reduced medium. The stirring speed was 500 rev/min, gas was sparged at 200 ml/min, the temperature was 37°C and the pH was controlled by the addition of sterile 2 mol/l KOH.

The concentration of carbon dioxide in the gas phase was controlled by adjusting the flow rates of CO_2 and N_2 in the influent gas, and was then determined by gas chromatography of the effluent gas. It has been demonstrated (Alford 1976; Yagi & Yoshida 1977) that determination of pCO_2 in the exhaust gas gives an excellent approximation to the partial pressure of dissolved CO_2 ($pCO_{2(aq)}$) in the liquid phase, eliminating the need for the more technically demanding measurement of the latter. Our own studies (Dixon, unpublished observations) indicate that under the stated conditions of pH, temperature, rates of stirring and sparging, and in the absence of added carbonic anhydrase, following a step change in the pCO_2 in the gas phase, the $t_{1/2}$ for the attainment of equilibrium between this and $pCO_{2(aq)}$ (as judged by the inorganic carbon content of the liquid phase) was less than 5 min.

ESTIMATION OF GROWTH

Growth was estimated by the measurement of the optical density, at 680 nm, of samples removed from the fermentation vessel. With a path length of 1 cm, 1 O.D. unit corresponded to 320 mg dry weight/l.

ESTIMATION OF FERMENTATION PRODUCTS

The end-products of fermentation were estimated by the means of gas chromatography (Stephens *et al.* 1985). Pentan-3-one was employed as internal standard. Glucose was estimated using Sigma enzyme kit No. 510. DL-Lactate was estimated by acidifying a 0·5 ml sample with one drop of concentrated HCl; 0·5 ml BF_3 in methanol was then added and the samples incubated overnight at 37°C. The samples were then extracted with 0·5 ml $CHCl_3$ and the methyl lactate in the $CHCl_3$ extract determined by gas chromatography (Holdeman & Moore 1975).

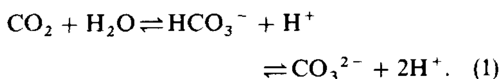
CHEMICALS

These were obtained from Sigma and were of analytical grade unless otherwise stated. Water was singly distilled in an all-glass apparatus.

THEORETICAL

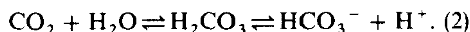
'CO_2' concentrations

Although the partial pressure of CO_2 in the gas phase may be held constant, the ratios of the different possible species of 'CO_2' in the aqueous phase will vary as a function of the pH and other factors. Since CO_2 can hydrate and dissociate with water, the reaction scheme may be written as



$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+. \quad (1)$$

In addition it has recently been proposed that small concentrations of dimeric hydrogen carbonate ions ($H_3C_2O_6^-$) exist near neutral pH (Covington 1985). Since the concentration of this species is negligible, however, such ions will not be considered in the following. At pH values less than 8, the concentration of carbonate ions may be neglected (Yagi & Yoshida 1977) and only the following reactions need to be considered



$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+. \quad (2)$$

Dissolved CO_2 concentration. The concentration of CO_2 in solution ($[CO_2]_{aq}$) is normally expressed by Henry's law (Butler 1982).

$$[CO_2]_{aq} = K_H pCO_2 \quad (3)$$

where K_H is the Henry's law constant (in units of mol/l/atm) and pCO_2 is the partial pressure of CO_2 in the gas phase (in atmospheres). Since our cultures were grown under atmospheric pressure, the proportionality of solubility and partial pressures (Henry's law) may be assumed without introducing appreciable errors (Schumpe *et al.* 1982).

At a temperature of 37°C, $K_H = 10^{-1.61}$ (Butler 1982) where the $[CO_2]$ is expressed in molar terms. Thus to obtain $[CO_2]$ in millimolar terms, $K_H = 10^{1.39}$. Hence

$$[CO_2]_{aq} = 10^{1.39} \times pCO_2 = 24.6 \times pCO_2.$$

In other words, when $pCO_2 = 1$ atm, the concentration of dissolved CO_2 = 24·6 mmol/l.

Bicarbonate concentration in a pH-controlled culture at 37°C with a gas phase of constant pCO_2. The equilibrium between CO_2 and HCO_3 is defined by a 'hybrid' equilibrium constant K_1' (Butler 1982) where

$$K_1' = \frac{10^{-pH}.[HCO_3^-]}{[CO_2]}. \quad (4)$$

From eqn (4) it follows that

$$\log_{10}[HCO_3^-] = pH - pK_1' + \log_{10}[CO_2]. \quad (5)$$

pK_1' is related to the thermodynamic pK of the reaction $pK_1°$ and the ionic strength *I* by

$$pK_1' + pK_1° - 0.5f(I) - bI. \quad (6)$$

At 37°C $pK_1° = 6.305$ and *b* takes the value 0·10 (Butler 1982).

From Davies' equation (Butler 1982)

$$f(I) = [I^{1/2}/(1 + I^{1/2}) - 0.21] \times [(298/(T + 273)]^{2/3} \quad (7)$$

where *T* is the temperature in °C. *I* is the ionic strength of the medium and is given by

$$I = 1/2 \sum c_i z_i^2 \quad (8)$$

where c_i = the concentration of ion *i* and z_i = the charge on ion *i*.

For EAA/FA medium $I = 0.124$ mol/l and hence for media with this ionic strength $f(I) = 0.049$.

To obtain the apparent $pK_{a,1}$ for the CO_2/HCO_3 equilibrium, we use (Butler 1982)

$$pK_{a,1} = pK^0_{a,1} - f(I) - bI. \quad (9)$$

Table 1. Bicarbonate concentrations in equilibrium with a CO_2 partial pressure of 1 atm at 37°C

pH	$[HCO_3^-]$ (mmol/l)
5·5	4·48
6·0	14·16
6·5	44·78
7·0	141·56
7·5	447·65
8·0	1415·58

Data were calculated from eqn (5), for a medium of ionic strength 0·124 mol/l, as described in the text.

Therefore from eqn (9), $pK_{a,1} = 6{\cdot}24$ and so, from eqn (5)

$$\log_{10}[HCO_3^-] = pH - 6{\cdot}24 + \log_{10}[CO_2]\ .$$

The relationship between pH and $[HCO_3^-]$ at 1 atm CO_2 (24·6 mmol/l $CO_{2(aq)}$) is shown in Table 1.

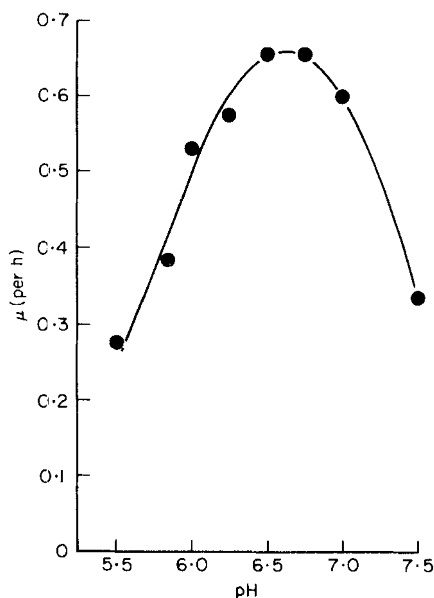


Fig. 1. The influence of pH on the growth of *Clostridium sporogenes* NCIB 8053. *Clostridium sporogenes* was grown in pH-controlled batch culture in essential amino acid/fatty acid medium sparged with CO_2 at 1 atm, as described in Materials and Methods.

Results

OPTIMUM pH

Since the concentrations of the different species of the various CO_2 couples referred to above vary with pH, the first step in this study was to determine the optimum pH for the growth of *Cl. sporogenes* NCIB 8053 in a defined medium. The pCO_2 in the gas phase was kept at a constant value of 1 atm whilst the pH was controlled at appropriate values in a series of different cultures. The medium used was EAA/FA and in this medium the optimal pH for growth was pH 6·5–6·75 (Fig. 1). All subsequent cultures were therefore grown at a controlled pH of 6·5.

EFFECTS OF pCO_2 ON GROWTH RATE

When grown in tryptone medium (see Materials and Methods) *Cl. sporogenes* NCIB 8053 grew more rapidly than in EAA/FA medium and at a rate that was independent of the pCO_2 (Fig. 2). Similarly the organism displayed no absolute requirement for CO_2 when grown in MACC medium; however, there was an optimal pCO_2 of some 0·45 atm ($[CO_2]_{aq}$ = 10·8 mmol/l) with higher pCO_2 values serving to decrease the observed growth rate (Fig. 3). By contrast, *Cl. sporogenes* did display an absolute requirement for CO_2 when grown in EAA/FA medium or in valine/proline medium (Figs 4 and 5), and once again the growth rate was inhibited at high pCO_2 values. The requirement for CO_2 in the latter media but not in MACC or tryptone media is consistent with the finding that *Cl. sporogenes* can produce certain L-amino acids by reductive carboxylation and transamination, as described elsewhere (Monticello *et al.* 1984; Lovitt *et al.* 1987b).

Figure 6 shows some of the major metabolic pathways of proteolytic clostridia in which CO_2 is known or thought to be involved (Andrew & Morris 1965; Gottschalk 1979; Monticello & Costilow 1982; Monticello *et al.* 1984; Lovitt *et al.* 1987a). It was initially argued that by providing 4-carbon-containing skeletons it might be possible to remove the ability of CO_2 to stimulate growth in MACC medium. As shown in Fig. 7, however, the growth of *Cl. sporogenes* on malate did not exhibit a requirement for CO_2 even in EAA/FA medium. Since it is known that

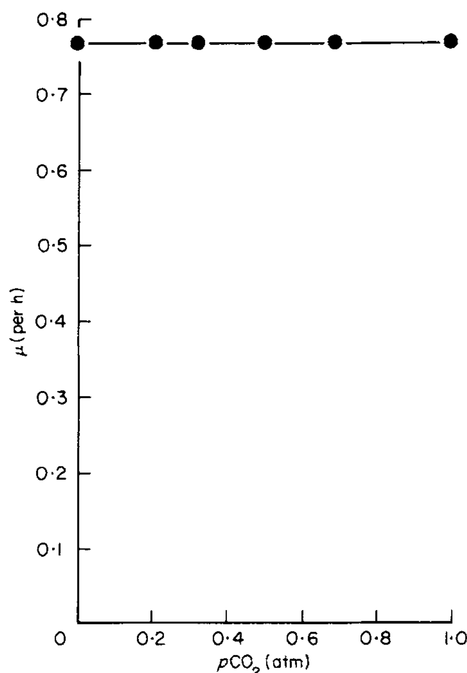


Fig. 2. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053. *Clostridium sporogenes* was grown in batch culture in tryptone medium, as described in Materials and Methods. Cultures were sparged with gas containing the appropriate partial pressure of CO_2.

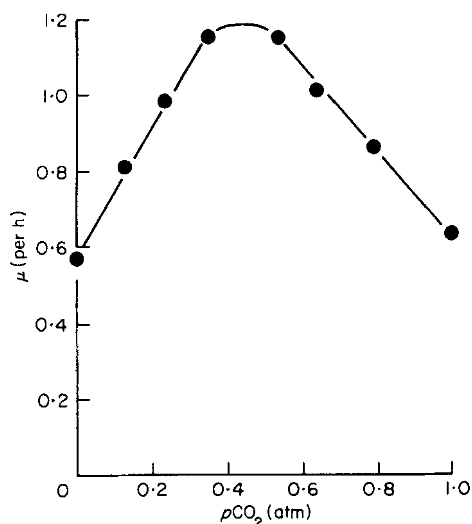


Fig. 3. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053 in amino acid complete medium. The organism was grown in batch culture as described in the legend to Fig. 2.

Cl. sporogenes can synthesize branched-chain amino acids by alternative routes in glucose-free media (Monticello & Costilow 1982; Monticello *et al.* 1984), it would appear that the substitution of glucose by malate causes the cells to induce amino acid biosynthetic pathways whose operation is independent of exogenous CO_2. Cells did not grow on malate in EAA/FA medium from which the fatty acids had been omitted (data not shown). At high pCO_2 values, CO_2 remained slightly inhibitory (Fig. 7).

The inhibitory effect of CO_2 on the growth of this organism might be due to one or more of the following: (1) feedback inhibition of the decarboxylation reactions occurring in the free energy-conserving metabolic pathways; (2) inhibition of the decarboxylation reactions involved in the synthesis of amino acids; or (3) alteration of the energetics of cell growth in a way similar to that proposed for *Klebsiella aerogenes* (Teixeira de Mattos *et al.* 1984), i.e. by 'metabolic uncoupling'. That CO_2 is likely to exert a major influence on the decarboxylation reactions involved in the synthesis of amino acids is indicated by the absence of growth inhibition at high pCO_2 values in the tryptone medium. To investigate the various possibilities,

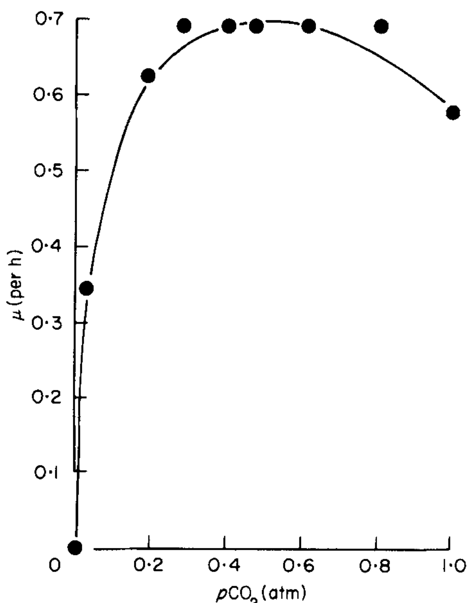


Fig. 4. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053 in essential amino acid/fatty acid medium. The organism was grown in batch culture as described in the legend to Fig. 2.

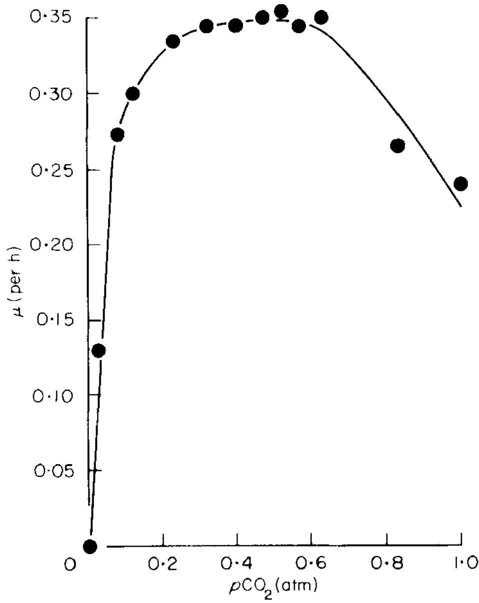


Fig. 5. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053 in valine/proline medium. The organism was grown in batch culture as described in the legend to Fig. 2.

we studied the effects of pCO_2 on the fermentation end-products produced by this organism in batch culture.

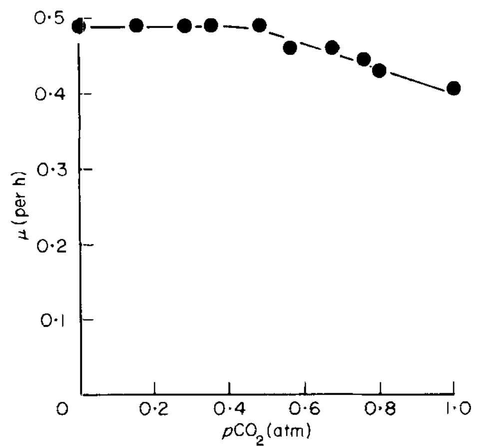


Fig. 7. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053 in malate medium. The organism was grown as described in the legend to Fig. 2.

EFFECT OF pCO_2 ON FERMENTATION PRODUCTS

The nature of the fermentation products produced by *Cl. sporogenes* NCIB 8053 growing in MACC depended significantly upon the pCO_2 (Fig. 8). As the pCO_2 increased, lactate

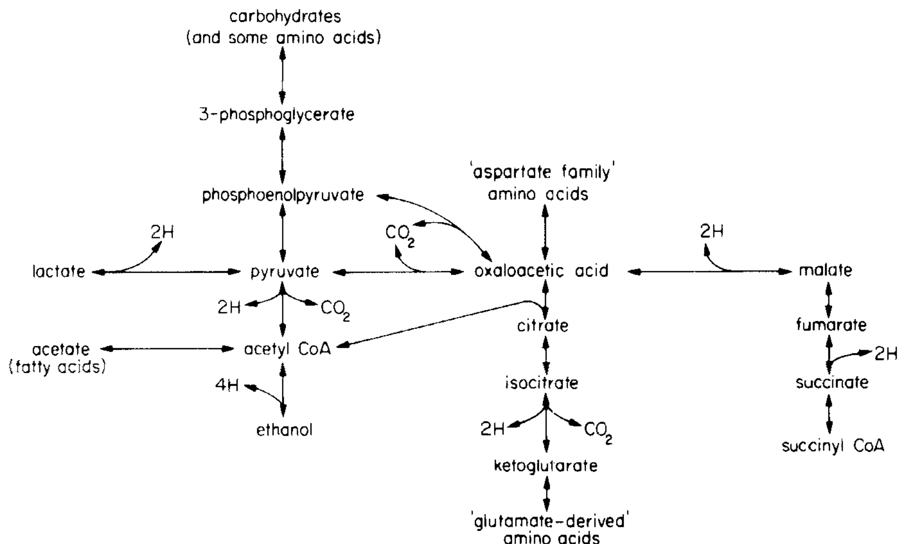


Fig. 6. Metabolic pathways implicated in the metabolism of CO_2 by *Clostridium sporogenes*. The evidence for the operation of enzymes in these pathways lies in the references cited in the text, except that for pyruvate carboxylase and isocitrate dehydrogenase which is from unpublished results of N.M. Dixon obtained using methods described by Scrutton (1971).

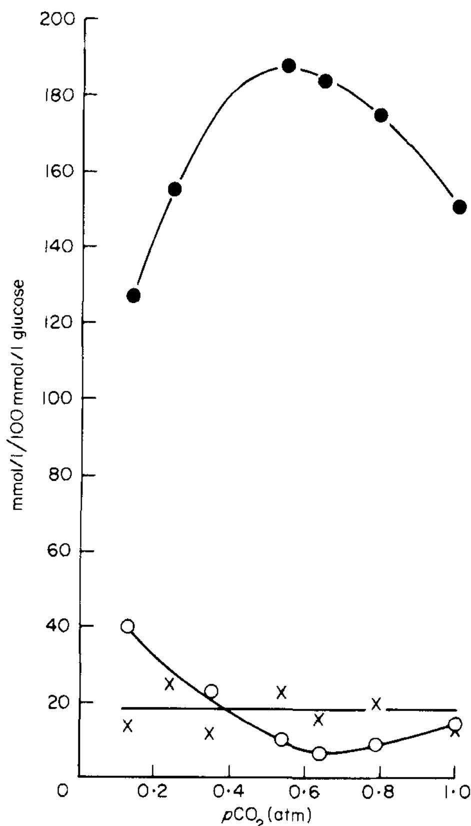


Fig. 8. The effect of pCO_2 on the fermentation products of *Clostridium sporogenes* NCIB 8053. *Clostridium sporogenes* was grown in amino acid complete medium as described in the legend to Fig. 2 and the fermentation products were estimated as described in Materials and Methods. ●, Acetate; ○, lactate; ×, ethanol.

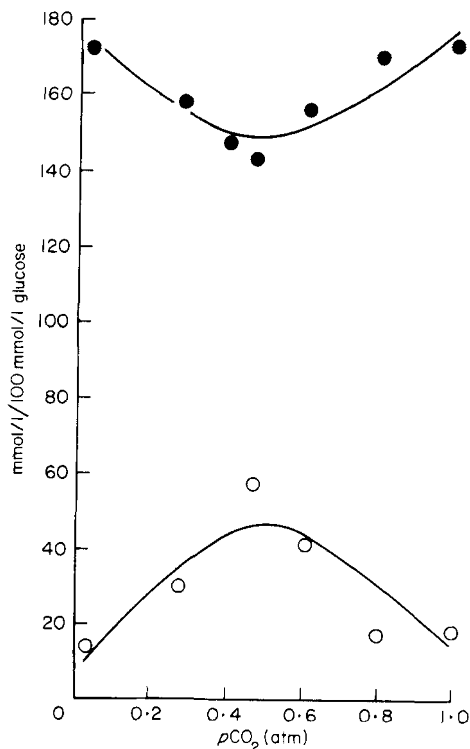


Fig. 9. The effect of pCO_2 on the fermentation products of *Clostridium sporogenes* NCIB 8053 grown in essential amino acid/fatty acid medium. The organism was grown and the fermentation products were estimated as described in the legend to Fig. 8. ●, Acetate; ○, ethanol.

production first decreased and then increased. This may be explained by assuming that at low pCO_2 there is a significant decrease in the rate of carboxylation reactions (Fig. 6), which will tend to lead to an increase in the pyruvate levels. To decrease the internal pyruvate concentration and remove excess reducing equivalents lactate is produced. As the pCO_2 increases, the carboxylation reactions are able to proceed more readily with a concomitant decrease in the pyruvate pool and hence less lactate production. At high values of pCO_2, decarboxylation reactions are inhibited (e.g. pyruvate to acetyl-CoA, Fig. 6) and thus acetate production is inhibited. Since acetate production is associated with a greater yield of ATP than are the other catabolic pathways (Thauer *et al.* 1977; Morris 1986) it is to be expected, and is found (cf. Figs 3 and 8), that the growth rate is rather closely mirrored in the proportional yield of acetate.

A shift in the proportion of the fermentation products was also observed when *Cl. sporogenes* NCIB 8053 was grown in EAA/FA medium (Fig. 9); in this case, however, the shifts were relatively small and, except when growth was strongly limited by pCO_2, were similarly related to growth rate.

APPLICATION OF THE METABOLIC CONTROL THEORY

When considering the control of metabolic pathways, it is traditional to ask the question

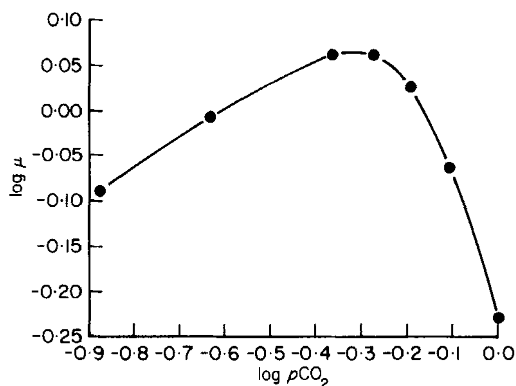


Fig. 10. The influence of pCO_2 on the growth of *Clostridium sporogenes* NCIB 8053 in amino acid complete medium. The data are those of Fig. 3 replotted on double logarithmic scales to illustrate the means by which one obtains control coefficients as the slope of such a plot.

'which step is rate-limiting?', when referring to enzymes in pathways, or 'what is rate-limiting?', when referring to pathway substrates or products. For instance, one might state, in traditional terms, that at high pCO_2 values the decarboxylation reactions are rate-limiting to the growth of *Cl. sporogenes*, and at low pCO_2 values it is the carboxylation reactions that are rate-limiting. Likewise, substrates or products may also be said to be limiting to growth, either through an insufficiently large concentration or due to an inhibitory concentration. CO_2 is something of a special case as it is both a substrate and a product of the metabolism of this organism (Fig. 6).

However, the question that should more properly be asked of a parameter is '*how* rate-limiting is it?' and it has become apparent that the appropriate means with which quantitatively to approach such a question are in terms of the metabolic control theory (see e.g. Kacser & Burns 1973; Kell & Westerhoff 1986a, 1986b). This theory derives from the general formalism of sensitivity analysis (Cruz 1973), in which the sensitivity of any variable to any parameter is expressed as a 'sensitivity coefficient' or a 'control coefficient'. The control coefficient of an enzyme, which expresses in quantitative terms the degree to which it may be rate-limiting to the flux through a metabolic pathway, is defined (e.g. Kell & Westerhoff 1986b) as

$$C^J i = ((\mathrm{d}J/\mathrm{d}e_i) \cdot (e_i/J))_{ss} = (\mathrm{d} \log |J| / \mathrm{d} \log e_i)_{ss} \quad (10)$$

where $C^J i$ is the control coefficient of enzyme i, J is the flux through the pathway, e_i is the concentration of enzyme i and ss denotes steady state. It should be noted that the differentials apply strictly to infinitesimal changes in the parameter and variable studied, but, particularly to accommodate values of the parameter equal to zero, it is permissible to use the (small but finite) values of the changes themselves (i.e. δJ, δe_i) to calculate the control coefficients.

By applying eqn (10) to the effect of pCO_2 on growth we may define the control coefficient for the effect of pCO_2 on the growth rate, $C^{\mu}_{CO_2}$, as

$$C^{\mu}_{CO_2} = ((\mathrm{d}\mu/\mathrm{d}pCO_2) \cdot (pCO_2/\mu))_{ss}. \quad (11)$$

The control coefficient for the influence of pCO_2 on the growth rate is given (see eqns 10 and 11) by the slope of a plot of log μ/log pCO_2; Fig. 10 shows such a plot for the growth of *Cl. sporogenes* NCIB 8053 in MACC medium, using the data given in Fig. 3.

In MACC medium the growth of *Cl. sporogenes* was most rate-limited by CO_2 at low pCO_2 values, but it would not be correct to state that growth was completely rate-limited by CO_2. This was also indicated by the ability of *Cl. sporogenes* to grow in the absence of CO_2; under these conditions the maximum control coefficient, $C^{\mu}_{CO_2} = 0·6$ (Fig. 11). $C^{\mu}_{CO_2}$ decreases as pCO_2 increases and hence CO_2 becomes less rate-limiting, until $C^{\mu}_{CO_2}$ becomes $= 0$ at the optimal pCO_2. As pCO_2 increases beyond its optimal value, CO_2 becomes inhibitory to growth rate and $C^{\mu}_{CO_2}$ attains a value of $-1·0$ at pCO_2 values exceeding 0·8 atm. When *Cl. sporogenes* was grown in tryptone medium CO_2 did not exert any influence on the growth rate and hence the control coefficient $= 0$ under all conditions.

In either EAA/FA medium or valine/proline medium there was an absolute requirement for CO_2 for the growth of *Cl. sporogenes*, hence the control coefficient $C^{\mu}_{CO_2} = 1$, and therefore CO_2 may be said to be 'completely rate-limiting for growth' (Figs 12 and 13). As the pCO_2 increases, the value of $C^{\mu}_{CO_2}$ decreases and hence, as before, CO_2 becomes less rate-limiting until the optimal pCO_2 is reached, where $C^{\mu}_{CO_2} = 0$. As the pCO_2 increases above its optimal value, CO_2 becomes

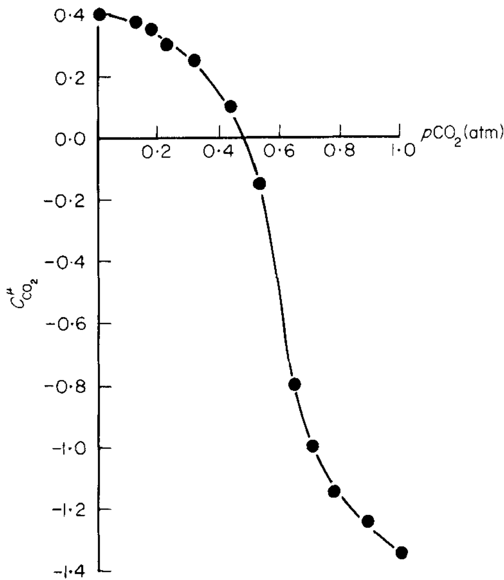


Fig. 11. The control coefficient $C^{\mu}_{CO_2}$ of pCO_2 on growth. *Clostridium sporogenes* NCIB 8053 was grown in amino acid complete medium, as described in the legend to Fig. 2. The control coefficients were calculated using $\delta\mu$ and δCO_2 values, obtained from Fig. 3, as described in the Results.

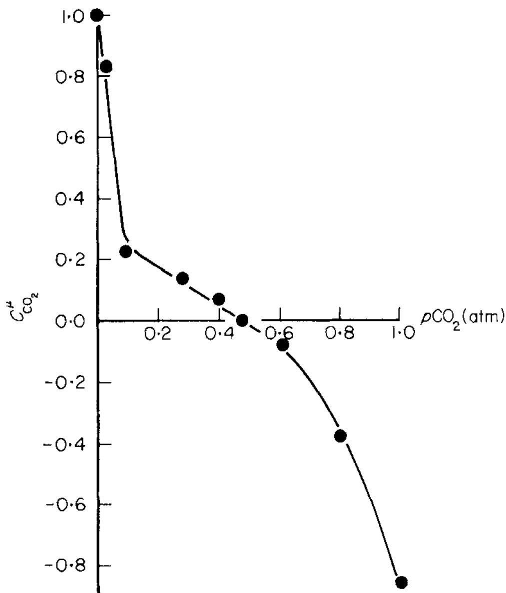


Fig. 12. The effect of pCO_2 on the control coefficient of growth, $C^{\mu}_{CO_2}$, of *Clostridium sporogenes* grown in essential amino acid/fatty acid medium. The control coefficients were calculated as described in the legend to Fig. 11, using the values of $\delta\mu$ and δCO_2 derived from Fig. 4.

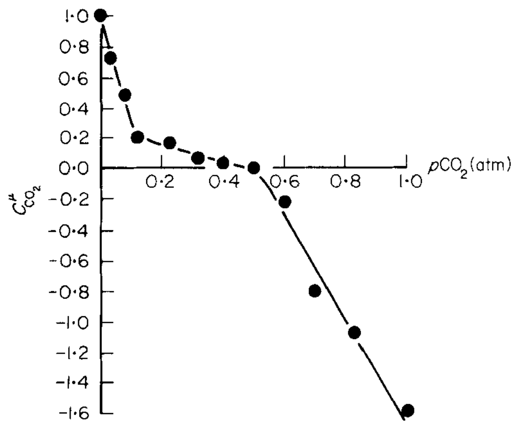


Fig. 13. The effect of pCO_2 on the control coefficient of growth, $C^{\mu}_{CO_2}$, of *Clostridium sporogenes* grown in valine/proline medium. The control coefficients were calculated as described in the legend to Fig. 11, using the values of $\delta\mu$ and δCO_2 derived from Fig. 5.

inhibitory, indicated by the increasingly negative values of $C^{\mu}_{CO_2}$. The changes of $C^{\mu}_{CO_2}$ as a function of CO_2 at values of pCO_2 below the optimum are identical for both EAA/FA and valine/proline media, suggesting that CO_2 may be acting to stimulate growth by the same mechanism in each case.

The effect of pCO_2 on its control coefficients for growth rate follows a different pattern when *Cl. sporogenes* is grown in malate medium (Fig. 14). For pCO_2 values below 0·4 atm CO_2 has no effect on growth and hence $C^{\mu}_{CO_2} = 0$; above

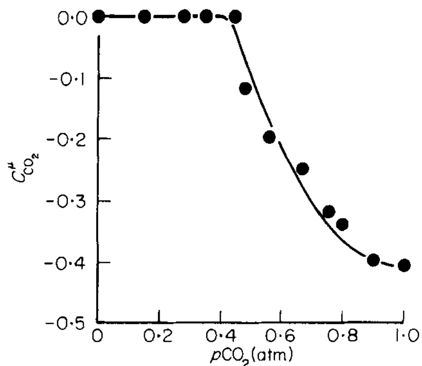


Fig. 14. The effect of pCO_2 on the control coefficient of growth, $C^{\mu}_{CO_2}$, of *Clostridium sporogenes* grown in malate medium. The control coefficients were calculated as described in the legend to Fig. 11, using the values of $\delta\mu$ and δCO_2 derived from Fig. 7.

0·4 atm CO_2 becomes increasingly inhibitory and $C^{\mu}_{CO_2}$ assumes increasingly negative values.

Discussion

The following evidence leads us to conclude not only that *Cl. sporogenes* is able to produce L-amino acids *via* reductive carboxylation and subsequent transamination of fatty acid precursors containing one less carbon atom than the corresponding L-amino acid (Monticello *et al.* 1984; Lovitt *et al.* 1987a, 1987b), but also that this pathway represents the *sole* reason for the requirement by this organism for CO_2. (1) There was an absolute requirement for CO_2 in both EAA/FA medium and valine/proline medium; (2) there was no CO_2 requirement in any of the other media. When *Cl. sporogenes* NCIB 8053 was provided with a supply of exogenous amino acids, i.e. in the tryptone medium, the organism is not required to synthesize any amino acids and hence CO_2 had no effect on growth rate (see also Reilly 1980). *Clostridium sporogenes* NCIB 8053 did not display an absolute requirement for CO_2 when grown in MACC medium, but continued to synthesize L-amino acids *via* CO_2-requiring reactions. This was demonstrated by the stimulation of growth by increasing pCO_2. It was mentioned above that Gladstone *et al.* (1935) had indicated that this organism displays an absolute requirement for CO_2, even in a relatively complex medium. Close scrutiny of this paper, however, indicates that the media used lacked isoleucine or a means of making it, consistent with the present data and interpretation.

The effects of CO_2 manifested in the alteration of the fermentation pattern displayed by *Cl. sporogenes* were presumably brought about by the inhibition of carboxylation reactions (due to lack of CO_2 at low pCO_2 values) and of decarboxylation reactions (due to inhibition at high pCO_2 values).

Analysis of the results in terms of the metabolic control theory gives a quantitative description of how much effect pCO_2 has on the growth rate of *Cl. sporogenes* NCIB 8053 in the various media. In EAA/FA and valine/proline media, where there is an absolute CO_2 requirement, CO_2 is the sole limiting factor ($C^{\mu}_{CO_2} = 1$ at 0 atm CO_2) as may have been expected. As pCO_2 increases, CO_2 becomes less rate-limiting to growth in MACC, EAA/FA and valine/proline media, until the optimal pCO_2 is reached, where $C^{\mu}_{CO_2} = 0$. At pCO_2 values above the optimal pCO_2, CO_2 becomes increasingly inhibitory to growth. The control coefficient for growth in tryptone medium = 0, i.e. pCO_2 has no effect on growth. Similarly for growth in malate medium, pCO_2 has no effect on growth until at pCO_2 values greater than 0·4 atm where it becomes increasingly inhibitory.

In conclusion, we have established how pCO_2 affects the growth and fermentation pattern of *Cl. sporogenes* NCIB 8053 and have demonstrated a requirement for CO_2 in certain media and the ability to over-ride this requirement by the provision of amino acid precursors from other sources, while the extent to which CO_2 is rate-limiting has been expressed in quantitative terms by the application of the metabolic control theory. Future work will be directed towards a quantitative study of the enzymatic basis for the effects of CO_2 on the growth of this organism in continuous culture.

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