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Growth energetics of *Clostridium sporogenes* NCIB 8053: modulation by CO_2

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The effects of the partial pressure of carbon dioxide on the growth energetics of *Clostridium sporogenes* NCIB 8053 grown in chemostat culture were investigated in defined minimal media. Both the 'maintenance' requirements and the growth yield coefficients were dependent upon the partial pressure of carbon dioxide in otherwise glucose-limited cultures. Since growth yield coefficients decreased along with the apparent 'maintenance' requirements in essential amino acid/fatty acid medium when the partial pressure of carbon dioxide was increased above 0·5 atm, the occurrence of some type of metabolic uncoupling seemed likely. By contrast, when the organism was grown in amino acid complete medium both the maintenance requirements and the growth yield coefficients were increased when the partial pressure of carbon dioxide was raised above 0·5 atm partial pressure of carbon dioxide, suggesting an increased efficiency of growth. A futile cycle involving carbon dioxide is proposed as a factor contributing to the variable extent of free energy dissipation within this organism.

The clostridia are a diverse group of spore-forming bacteria that are distinguished on the basis of their ability to ferment different carbon sources. This group contains both saccharolytic and proteolytic organisms. The biochemistry and physiology of the former have been the more widely studied, and they have recently been re-examined in detail because of their ability to produce solvents and organic acids from carbohydrate feedstocks (Zeikus 1980; 1985; Morris 1982). Similarly, the proteolytic clostridia which ferment amino acids have also been the subject of renewed interest, although attention has been focused more upon the potentially useful enzymes that these organisms produce (Lovitt *et al.* 1987a). Mindful of the possible biotechnological importance of this genus in microbial biotransformations generally (Morris 1982), and with a view to effecting the bioconversion of certain xenobiotics to fine chemicals, we have initiated a study of the reactions catalysed by such enzymes (James *et al.* 1988; Lovitt *et al.* 1987a).

In addition, proteolytic clostridia have been studied because they are a major agent in the spoilage of neutral and low-acid canned foods on account of their ability to form heat-resistant spores (Frazier & Westhoff 1978). *Clostridium sporogenes*, in particular, has been studied because of its resemblance to proteolytic, toxin-producing strains of *Clostridium botulinum*. Most studies on *Cl. sporogenes*, however, have been carried out in complex media (Lovitt *et al.* 1987b).

Defined media were recently described for the growth of *Cl. sporogenes* NCIB 8053 (Lovitt

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et al. 1987b), allowing quantitative studies to be made of the physiology of this organism. After the development of these media the growth energetics of *Cl. sporogenes* were investigated in glucose- or L-valine-limited chemostat cultures (Lovitt *et al.* 1987c). The gas phase during these chemostat studies was 1 atm CO_2, since it had been shown in an early report that CO_2 is either required for, or stimulates the growth of *Cl. sporogenes* (Gladstone *et al.* 1935). No attempt was made in this work to optimise the p_{CO_2} under which the organisms were grown, and a study was therefore undertaken of the effects of the partial pressure of CO_2 (p_{CO_2}) on the growth and metabolic behaviour of *Cl. sporogenes* in batch cultures (Dixon *et al.* 1987). High p_{CO_2} proved to be inhibitory to growth except in rich complex media, whilst the absolute CO_2 requirement displayed by this organism in defined minimal medium was shown to be due to the involvement of CO_2 in the synthesis of branched-chain amino acids. It was noted that the relative proportion of glucose metabolized to acetate, as the p_{CO_2} was increased, closely mirrored the corresponding changes in growth rate. This might be expected, since acetate production is associated with a greater yield of ATP than may be obtained from the formation of any other product of glucose dissimilation formed by this organism (Thauer *et al.* 1977; Morris 1986).

One possible effect of a supraoptimal p_{CO_2} on *Cl. sporogenes* might be that observed in an anaerobic glucose-limited chemostat culture of *Klebsiella aerogenes*, where it was demonstrated that increased concentrations of metabolically produced CO_2 were accompanied by a lowering of the growth yield coefficient (Teixeira de Mattos *et al.* 1984). In this case, the mechanism proposed was that a futile substrate cycle, dissipating free energy, was stimulated by an increased p_{CO_2}, such that carboxylation and decarboxylation reactions were enhanced, with resultant net ATP hydrolysis (Teixeira de Mattos *et al.* 1984).

Clostridium sporogenes has been shown to possess enzymes typical of organisms performing glycolysis by the Embden–Meyerhof–Parnas pathway (Lovitt *et al.* 1987c), allowing an accurate assessment of the energetics of the growth of the organism. To investigate the effect of CO_2 on the energetics and kinetics of the growth of *Cl. sporogenes* under more rigorously controlled conditions, we wished to study this organism further in chemostat cultures. The results of these investigations are presented.

Materials and Methods

CHEMICALS

These were obtained from Sigma and were of analytical grade unless otherwise stated. Single glass-distilled water was used.

ORGANISM

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

GROWTH MEDIA

All growth media were based upon a low-phosphate basal medium (LPBM), as described previously (Lovitt *et al.* 1987c), containing (g/l unless otherwise 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*-amino benzoic acid, 5; nicotinic acid, 500.

The amino acid complete medium (MACC) (Lovitt *et al.* 1987c) was LPBM supplemented with the following L-amino acids at these final concentrations (mmol/l): glycine, 3; valine, 2; isoleucine, 2; 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. Both of the above media contained (final concentrations in mmol/l) L-proline, 20; and glucose, 10. Growth

Table 1. The effect of dilution rate of *Clostridium sporogenes* in EAA/FA medium in glucose-limited chemostat culture under 0·10 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·038	26·2	0·75	18·27	3·83	95
0·040	26·9	1·91	17·18	3·72	96
0·080	35·8	1·88	17·14	3·71	95
0·110	39·7	1·97	17·14	3·71	96
0·140	41·0	1·96	18·52	3·85	102
0·140	46·1	1·30	18·31	3·83	98
0·180	49·9	1·87	17·96	3·80	99
				Mean 3·78	

* The organism was grown in essential amino acid/fatty acid medium, containing 10 mmol/l glucose, pH 6·5, at 37°C, stirring rate 500 rev/min and gas flow rate of 200 ml/min, as described in the Materials and Methods section.

$Y_{x/s}$, specific yield of biomass, i.e. g dry wt of cells produced/mol of glucose utilized. The $ATP_{glucose}$ (mol ATP produced/mol glucose dissimilated) figure assumes that no ATP is formed due to the reduction of proline.

The fermentation products are mmol/l per 10 mmol/l of glucose utilized.

The recoveries are the recovery of glucose carbon in the fermentation products, assuming 1 mol CO_2 is produced during the production of 1 mol ethanol or acetate. The percentage of carbon in the biomass was 48% (R. W. Lovitt unpublished results), established using a total organic carbon analyser (Beckman 915-B) (Gottschal & Morris 1981).

of the organism in these media in chemostat cultures was glucose-limited. In each case, these media were pre-reduced by the addition of 3 mmol/l of L-cysteine and stored in a 20 l reservoir under a nitrogen headspace.

CULTIVATION OF ORGANISMS

Inocula were grown overnight in pressure tubes (Bellco, Vineland, NJ) as described by Lovitt *et al.* (1987c). An overnight culture (10 ml) was then injected into a fermentation vessel of 480 ml working volume (LH Engineering, Stoke Poges, Bucks), containing pre-reduced medium. Medium flow from the reservoir was controlled with either an LKB (Salsdon, Surrey) 2132 Microperpex pump, or an LKB 2120 Varioperpex pump. The stirring speed was 500 rev/min, the temperature was 37°C and the pH was maintained at 6·5 by the automatic addition of sterile 2 mol/l KOH. Gas was sparged at 200 ml/min; the steady-state concentration of carbon dioxide was controlled and measured gas chromatographically, as previously described (Dixon *et al.* 1987).

ESTIMATION OF GROWTH

Growth was estimated by the measurement of the optical density (O.D.), 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 means of gas liquid chromatography (Stephens *et al.* 1985). Pentan-3-one was employed as internal standard.

Results

EFFECTS OF p_{CO_2} ON THE GROWTH OF *Clostridium sporogenes* IN EAA/FA MEDIUM IN GLUCOSE-LIMITED CHEMOSTAT CULTURE

The growth energetics of *Cl. sporogenes* at different values of p_{CO_2} were studied in glucose-limited chemostat cultures. The steady-state criterion for all experiments was that the biomass content was unchanging after seven to ten volume changes following the shift to a new

Table 2. The effect of dilution rate on *Clostridium sporogenes* in EAA/FA medium in glucose-limited chemostat culture under 0·29 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·030	33·0	3·22	16·88	3·69	100
0·090	26·9	3·22	18·07	3·81	106
0·130	28·8	2·68	17·89	3·79	103
0·210	34·6	2·26	17·82	3·78	100
0·225	34·6	2·68	18·01	3·80	103
0·250	38·4	2·80	17·87	3·79	103
0·325	34·6	3·65	16·24	3·62	99
				Mean 3·75	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

dilution rate. The growth kinetic and energetic behaviour of this organism at $p_{CO_2} = 0{\cdot}1$ atm and at higher values of p_{CO_2} are tabulated in Tables 1–5. Acetate and ethanol were the only end products observed and no significant dependence of the proportion of the end products of glucose catabolism upon growth rate was discernible. In addition to this, there was no

Table 3. The effect of dilution rate on *Clostridium sporogenes* in EAA/FA medium in glucose-limited chemostat culture under 0·56 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·075	40·3	1·43	18·00	3·80	97
0·125	41·3	1·40	18·57	3·86	100
0·175	48·6	1·43	19·37	3·94	104
0·250	52·5	0·75	19·50	3·95	101
0·340	58·9	0·12	19·40	3·94	98
				Mean 3·90	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

Table 4. The effect of dilution rate on *Clostridium sporogenes* in EAA/FA medium in glucose-limited chemostat culture under 0·75 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·075	38·4	1·14	18·88	3·88	100
0·075	38·4	1·98	18·78	3·88	104
0·125	43·5	1·32	17·90	3·79	96
0·150	40·9	1·74	18·08	3·81	99
0·175	44·8	1·49	17·01	3·70	92
0·190	40·9	1·17	18·04	3·80	96
0·230	46·1	1·95	17·65	3·77	98
0·280	44·8	0·88	18·22	3·82	96
0·290	44·8	1·87	18·03	3·80	100
				Mean 3·81	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

Table 5. The effect of dilution rate on *Clostridium sporogenes* in EAA/FA medium in glucose-limited chemostat culture under 1·00 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·090	40·3	0·97	19·12	3·92	100
0·010	43·2	0·69	18·93	3·89	98
0·180	45·1	1·23	18·22	3·82	97
0·180	44·8	0·82	18·40	3·84	96
0·260	47·0	0·50	17·85	3·79	92
0·310	43·2	1·05	18·17	3·82	96
0·400	49·0	1·59	17·81	3·78	97
0·460	48·0	1·83	17·77	3·78	98
0·500	46·1	1·35	17·84	3·78	96
0·570	34·6	1·40	18·12	3·81	98
				Mean 3·82	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

noticeable relationship between the partial pressure of CO_2 in the headspace and the nature of the end products formed. The recovery of glucose carbon in the fermentation end products was close to 100% (Tables 1–5).

Figure 1 illustrates typical results for the specific rates of substrate consumption as a function of dilution rate, using information from Tables 1, 2 and 5. From the intercept of such plots with the ordinate it is possible to determine the so-called maintenance requirement (Pirt 1975; Tempest & Neijssel 1984). The maximum specific growth yield and maintenance coefficients, as calculated from the data in Tables 1–5, are shown in Figs 2a and 3a. In EAA/FA medium the maintenance requirement was significantly reduced as the p_{CO_2} was increased. The growth yield coefficient initially increased with increasing p_{CO_2}, up to an optimum p_{CO_2} of approximately 0·5 atm (Fig. 2a). The effect of p_{CO_2} on the growth yield of *Cl. sporogenes* in EAA/FA medium thus closely resembled its effect upon the growth rate of this organism in the same medium in batch cultures (Dixon *et al.* 1987).

EFFECTS OF p_{CO_2} ON THE GROWTH ENERGETICS OF *Clostridium sporogenes* IN MACC MEDIUM IN GLUCOSE-LIMITED CHEMOSTAT CULTURE

In previous work (Dixon *et al.* 1987) we established that the absolute requirement for CO_2 displayed by *Cl. sporogenes* in EAA/FA medium was due to its use in the biosynthesis of various branched-chain amino acids, a requirement not found in MACC medium. To establish the effect of CO_2 upon the growth of *Cl. sporogenes* under conditions in which CO_2 was not actually required for growth, a similar study to that carried out above was performed in MACC medium.

The results obtained for the growth of *Cl. sporogenes* in glucose-limited chemostat cultures in MACC medium are shown in Tables 6–10. As with the EAA/FA chemostat cultures, acetate and ethanol were the only end products observed. There did not appear to be any significant relationship between either dilution (growth) rate and end product formation nor between CO_2 partial pressure and the nature of the fermentation end products. As with the growth of this organism in EAA/FA medium, the recovery of glucose carbon in the end products was close to 100% (Tables 6–10).

The maximum specific growth yield and maintenance coefficients, as calculated from the results in Tables 6–10, are shown in Figs 2b and 3b. In MACC medium, as in EAA/FA medium, the maintenance requirement initially decreased with increasing p_{CO_2}; as the p_{CO_2} increased above 0·5 atm, however, the maintenance requirement also increased.

Discussion

Despite the existence of a number of reports on the effects of p_{CO_2} on various bacteria, mostly in

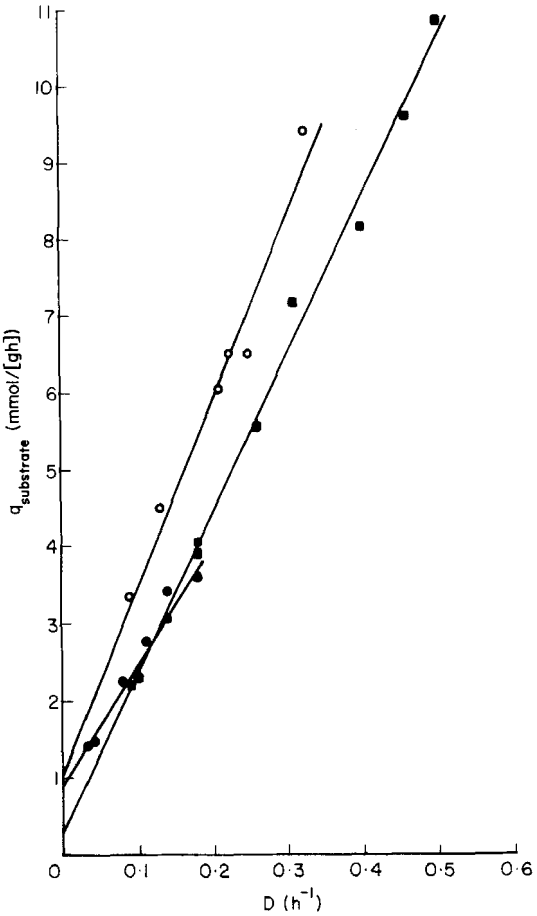

Fig. 1. Catabolic rates exhibited by *Clostridium sporogenes* grown in EAA/FA medium, at various p_{CO_2}. This figure is an example of a typical plot of $q_{substrate}$ vs dilution rate. The other plots of $q_{substrate}$ vs dilution rate are not given; however, they are all rectilinear. The values of $q_{substrate}$ were calculated from data in Tables 1, 2 and 5. ●, 0·10 atm p_{CO_2}; ○, 0·29 atm p_{CO_2}; ■, 1·0 atm p_{CO_2}.

relation to the preservation of foodstuffs (e.g. Clark & Lentz 1969; Spahl *et al.* 1981; Finne 1982; Blickstad & Molin 1983; Wang & Ogrydziak 1986), relatively few of these studies were carried out in defined media and in chemostat cultures. In an aerobic chemostat culture, the maximum specific growth rate of *Pseudomonas fragi* was decreased with increasing p_{CO_2} (Molin 1983). The effects of p_{CO_2} on the growth energetics of this organism, however, were not reported. During ethanol production by *Zymomonas mobilis* in continuous culture the growth yields, at high dilution rates, were reduced by increased p_{CO_2} (Nipkow *et al.* 1985). The product yields were not affected by p_{CO_2}, although the production rates were slightly increased by high p_{CO_2}. Increased concentrations of metabolically produced CO_2 were accompanied by a lowering of the growth yield coefficient of *Klebsiella aerogenes* (Teixeira de Mattos *et al.* 1984). In contrast to *Ps. fragi* and *Z. mobilis*, the dry weight and Y_{ATP} of *Cl. butyricum* were unaffected by values of p_{CO_2} between 0 and 1 atm (van Andel *et al.* 1985).

SOME RELEVANT CHEMOSTAT THEORY

Bacterial growth in chemostat culture is most often described in terms of Monod kinetics, though it was noted that Blackman kinetics can in fact generally provide a better fit of chemostat results (Dabes *et al.* 1973). The difference between the two formalisms is that Monod kinetics assume that one step (often the primary step; e.g. Button 1985) is rate-limiting, whilst Blackman kinetics assume that one or more steps are in turn rate-limiting as (in this case) the

Table 6. The effect of dilution rate on *Clostridium sporogenes* in MACC medium in glucose-limited chemostat culture under 0·10 atm p_{CO_2}*

		Fermentation products			
D/h	$Y_{x/s}$ (g/mol)	Ethanol (mmol/l)	Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·038	32·6	0·64	19·06	3·91	98
0·050	39·7	0·81	18·96	3·90	99
0·080	44·8	1·21	18·99	3·90	101
0·120	53·8	0·34	18·24	3·82	93
0·175	49·9	2·43	17·75	3·78	101
0·175	51·2	1·14	18·35	3·83	97
0·250	53·8	2·06	17·71	3·77	99
				Mean 3·84	

* The organism was grown in essential amino acid/fatty medium containing 10 mmol/l of glucose as described in the legend to Table 1.

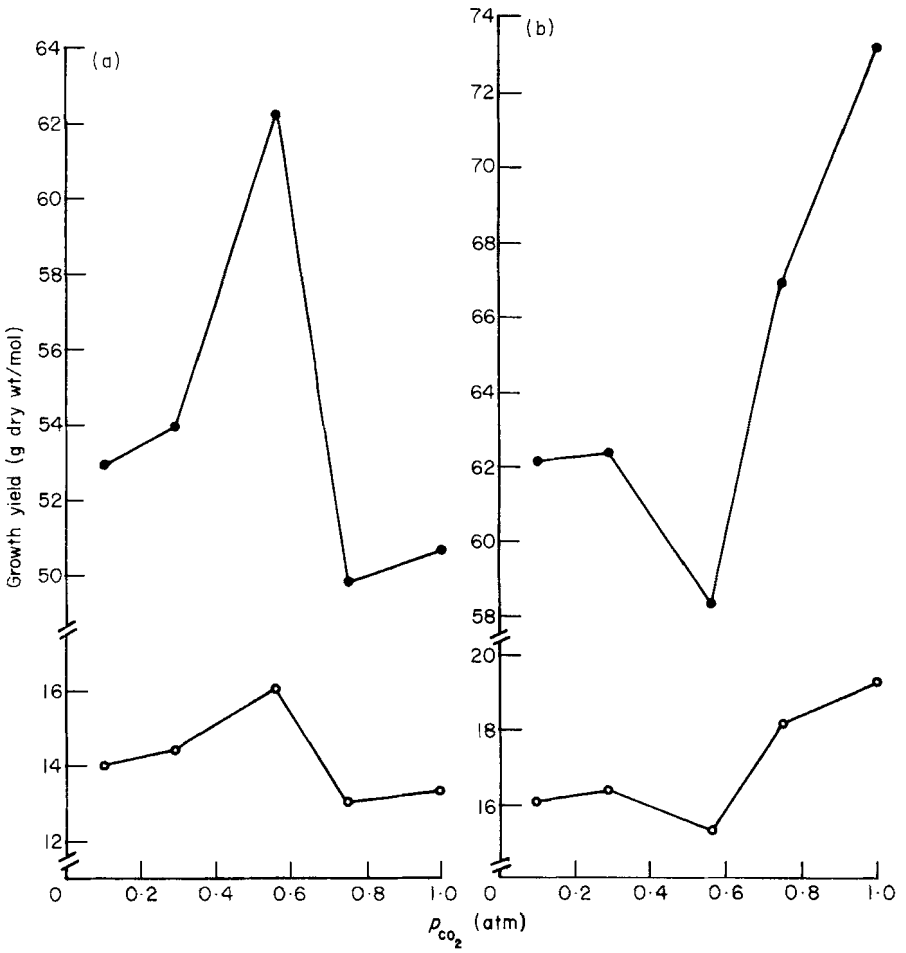

Fig. 2. Influence of p_{CO_2} on the growth yields of *Clostridium sporogenes* grown in either EAA/FA or MACC medium, as described in the legend to Table 1. a, The data for growth in EAA/FA medium were derived from Tables 1–5. ●, $Y_{glucose}^{max}$; ○, Y_{ATP}^{max}; b, the data for growth in MACC medium were derived from Tables 6–10. ●, $Y_{glucose}^{max}$; ○ Y_{ATP}^{max}.

Table 7. The effect of dilution rate on *Clostridium sporogenes* in MACC medium in glucose-limited chemostat culture under 0·29 atm p_{CO_2}*

		Fermentation products			
D/h	$Y_{x/s}$ (g/mol)	Ethanol (mmol/l)	Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·050	38·4	1·41	17·59	3·76	94
0·090	49·9	1·34	19·31	3·93	103
0·120	44·8	1·76	17·91	3·79	98
0·175	49·9	2·15	17·83	3·78	100
0·180	55·0	1·34	17·73	3·77	95
0·225	58·9	1·43	17·71	3·77	96
				Mean 3·80	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

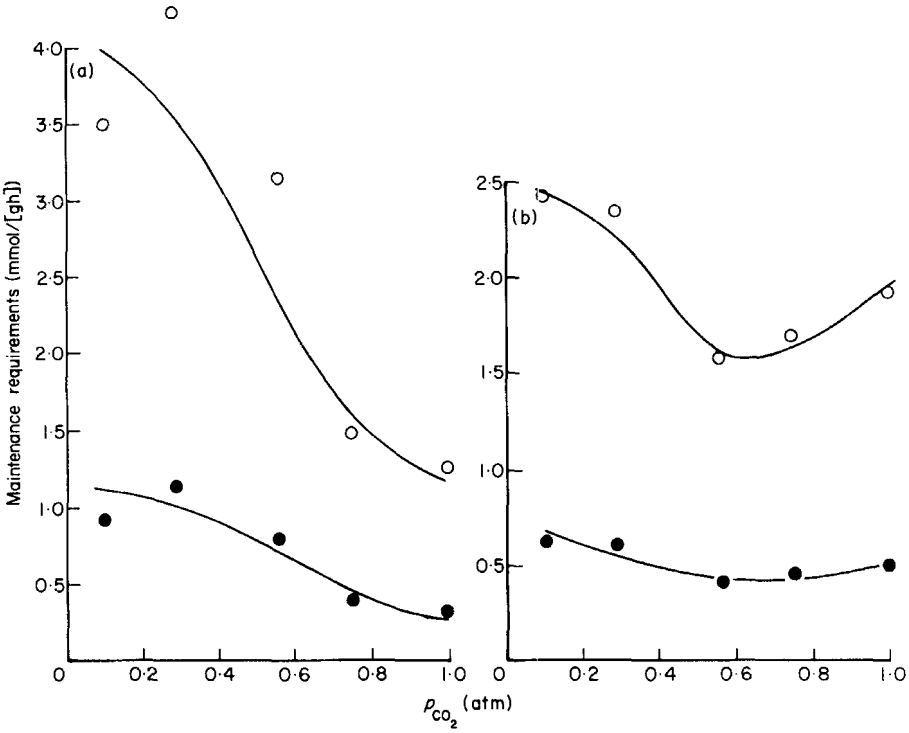

Fig. 3. Influence of p_{CO_2} on the maintenance requirements of *Cl. sporogenes* grown either in EAA/FA or MACC medium, as described in the legend to Table 1. a, The data for growth in EAA/FA medium were derived from Tables 1–5 and Fig. 2. ●, $m_{(qm)}$; ○, m_{ATP}; b, the data for growth in MACC medium were derived from Tables 6–10. ●, $m_{(qm)}$; ○, m_{ATP}.

dilution rate is increased, and thus more closely resembles the metabolic control analysis (Kell & Westerhoff 1986a;1986b; Kacser & Porteous 1987; Kell 1987; Westerhoff & van Dam 1987). The metabolic control analysis shows that in a metabolic system, no one step is generally rate-limiting; that all steps are to some extent rate-limiting or exert flux control; and that the degree of limitation depends upon both the environmental conditions and the relevant enzyme kinetic parameters. Both Blackman kinetics and the metabolic control theory make more realistic assumptions and thus provide more realistic descriptions of microbial behavi-

Table 8. The effect of dilution rate on *Clostridium sporogenes* in MACC medium in glucose-limited chemostat culture under 0·56 atm p_{CO_2}*

		Fermentation products			
D/h	$Y_{x/s}$ (g/mol)	Ethanol (mmol/l)	Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·104	46·1	1·26	17·31	3·73	93
0·117	49·9	2·61	17·80	3·78	102
0·175	51·2	2·30	17·90	3·79	101
0·250	52·5	0·84	18·84	3·88	98
0·360	55·0	0·77	18·23	3·82	95
0·420	55·0	0·67	17·87	3·79	93
				Mean 3·80	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

Table 9. The effect of dilution rate on *Clostridium sporogenes* in MACC medium in glucose-limited chemostat culture under 0·75 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·080	49·9	3·65	15·99	3·60	98
0·150	53·8	3·87	16·11	3·61	100
0·200	56·3	2·52	16·81	3·68	97
0·250	62·7	2·20	17·12	3·71	97
0·270	62·7	2·26	17·73	3·77	100
0·380	61·4	2·13	17·49	3·75	98
				Mean 3·69	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

our than do Monod kinetics. Indeed, it is worth noting that not all of Monod's classical data fit Monod kinetics perfectly (Dabes *et al.* 1973). The formalisms for both Monod and Blackman kinetics contain two kinetic constants, whilst that for so-called Best kinetics contains three constants and can thus provide a better fit of chemostat data than even the Blackman kinetics (Dabes *et al.* 1973; Koch 1985).

When describing bacterial growth the usual criterion of limitation is not a positive (and approximately proportional) effect of increasing the concentration of the limiting substrate on the steady-state biomass concentration, but either a nil effect of increasing all of the other medium components or a more-or-less proportional decrease in the steady-state biomass concentration when the reservoir concentration of the growth-limiting substrate is decreased (Tempest 1970). That the recovery of glucose was close to 100% indicates that even though the media used were defined minimal media for this organism (Lovitt *et al.* 1987b; 1987c), much of the carbon content of the biomass was derived from non-carbohydrate sources. Thus the cultures may be taken to be energy-limited (Tempest & Neijssel 1984). The biomass yields are expressed as growth yields ($Y_{x/s}$) with units of g dry weight per mol of energy source utilized; the maximum growth yields ($Y^{max}_{x/s}$) are the maximum achievable per mol of energy source and are obtained by extrapolating $Y_{x/s}$ values to infinite dilution rate. In the case of glucose-limited culture these yields become $Y_{glucose}$ and $Y^{max}_{glucose}$. Since the number of mols of ATP may vary even for a given energy source, yield coefficients such as $Y_{glucose}$ and $Y^{max}_{glucose}$ are usually converted to Y_{ATP} and

Table 10. The effect of dilution rate on *Clostridium sporogenes* in MACC medium in glucose-limited chemostat culture under 1·00 atm p_{CO_2}*

D/h	$Y_{x/s}$ (g/mol)	Fermentation products: Ethanol (mmol/l)	Fermentation products: Acetate (mmol/l)	$ATP_{glucose}$ (mol/mol)	Recoveries (%)
0·090	47·4	1·34	17·27	3·73	93
0·150	52·5	1·59	17·98	3·80	98
0·200	56·3	1·01	17·87	3·79	94
0·210	55·0	2·17	17·97	3·80	101
0·300	64·0	0·30	18·10	3·81	92
0·370	67·2	1·61	17·99	3·80	98
0·410	53·8	0·87	18·80	3·88	98
0·490	67·2	1·25	18·35	3·83	98
0·650	54·4	1·64	18·03	3·80	98
				Mean 3·80	

* The organism was grown in essential amino acid/fatty acid medium containing 10 mmol/l of glucose as described in the legend to Table 1.

Y^{max}_{ATP} respectively, which also allows comparison of the yield coefficients of growth on energy sources that produce differing quantities of ATP (mol/mol). *Clostridium sporogenes*, when fermenting glucose in the presence of proline, produces ATP predominantly via substrate level phosphorylation, as opposed to electron transport phosphorylation, hence Y_{ATP} may be calculated directly. Maintenance requirements serve to reduce the yield of biomass per mol of energy source or ATP. As maintenance requirements increase as a proportion of the total energy required (or D decreases) the amount of free energy available for biomass synthesis will tend to decrease, and hence so to will the yield coefficients.

That these kinetics are merely approximations to what actually occurs during microbial growth was demonstrated, for instance, by the effect of metabolically produced CO_2 on the growth of *Klebsiella aerogenes* (Teixeira de Mattos *et al.* 1984), and by the effects of p_{CO_2} on *Cl. sporogenes* reported above.

EFFECT OF p_{CO_2} ON THE MAINTENANCE REQUIREMENTS OF *Clostridium sporogenes*

In both the MACC and EAA/FA media, increasing the p_{CO_2} initially resulted in a corresponding decrease in the maintenance requirements of *Cl. sporogenes*. This organism is able to synthesize branched-chain amino acids via pathways containing carboxylation reactions (Monticello *et al.* 1984; Dixon *et al.* 1987; Lovitt *et al.* 1987b; 1987c), and so at low p_{CO_2} values these reactions may become restricted. To be able to grow in EAA/FA medium, *Cl. sporogenes* is obliged to produce branched-chain amino acids via such reductive carboxylation, and hence displays an absolute requirement for CO_2. All of the essential amino acids are provided in MACC medium and so the organism does not display an absolute requirement for CO_2, although amino acids are still produced by carboxylating pathways as indicated by the stimulation of growth rate by CO_2 (Dixon *et al.* 1987). Hence the maintenance requirements in EAA/FA medium may be expected to be greater, and more influenced by p_{CO_2}, than those observed in MACC medium. This is indeed the case (Fig. 3).

Above 0·5 atm p_{CO_2} in MACC medium, the maintenance requirements increase with increasing p_{CO_2}. This increase in maintenance requirements could be due to (1) the feedback inhibition of decarboxylation reactions involved in free energy conservation, (2) the feedback inhibition of amino acid-producing decarboxylation pathways, or (3) metabolic-uncoupling (see also Teixeira de Mattos *et al.* 1984). That this effect was not observed in EAA/FA medium could be due to the existence of a greater requirement for CO_2 in this medium than in MACC medium, such that the carboxylation reactions make a proportionally greater contribution to the maintenance requirements than do the decarboxylation reactions, so that the effect of CO_2 inhibition is overcome by the greater effect of CO_2 stimulation.

MECHANISMS OF METABOLIC UNCOUPLING

Current thinking admits at least four types of mechanism by which uncoupled growth may be explained: (1) variation of the ATP requirement for biomass formation accompanying changes in the anabolic routes employed—perhaps most graphically illustrated by experiments in which genetic manipulations that permitted an improvement in the efficiency of the route of ammonium assimilation in a methylotroph were directly accompanied by a concomitant increase in growth yield (Windass *et al.* 1980); (2) variation in the ATP yield of catabolism, wrought either by changes in fermentation pathways (e.g. de Vries *et al.* 1970, Thauer *et al.* 1977) or in the efficiency (number of sites) of electron transport phosphorylation (e.g. Jones 1977); (3) variation in the permeability of the bilayer portions of the bacterial cytoplasmic membrane to protons and to other ions and metabolites (Stouthamer 1979); (4) the presence of uncoupled ATP hydrolase activity in the cells, either directly or as a result of the operation of futile cycles (e.g. Senez 1962; Stouthamer & Bettenhausen 1977; Teixeira de Mattos *et al.* 1984). Further, as the formalisms of macroscopic non-equilibrium thermodynamics (Kedem & Caplan 1965; Nicolis & Prigogine 1977; Hill 1977) have been extended to the problems of microbial transport (Eddy 1980; Ahmed & Booth 1981), growth (Hellingwerf *et al.* 1982; Westerhoff *et al.* 1982) and energetics (Westerhoff & van Dam 1979; Harder *et al.* 1981; Westerhoff & van Dam 1987), it has been recognized (Kell & Westerhoff

1985; Pietrobon & Caplan 1985; Pietrobon 1986; Pietrobon *et al.* 1986; Zoratti *et al.* 1986) that at least four different kinds of slip may occur in principle *within* proton motivated membrane energy coupling systems themselves (pump slip and leak slip), in addition to the general membrane leakiness alluded to in (3) above. Thus, both the ionmotive sources and sinks may (1) themselves catalyse proton or substrate leakage across the cytoplasmic membrane and/or (2) carry out metabolic reactions, such as electron transport, in a fashion that is not, or is only lossely and variably, coupled to proton or to other ion transport processes.

One possible mechanism of CO_2-dependent metabolic uncoupling would be the futile cycle that was proposed for *Klebsiella aerogenes* (Teixeira de Mattos *et al.* 1984), as depicted below:

$$\text{phosphoenolpyruvate} + CO_2 \xrightarrow[\text{PEP carboxylase}]{} \text{oxaloacetate} + P_i$$

$$\text{oxaloacetate} + ATP \xrightarrow[\text{PEP carboxykinase}]{} \text{phosphoenolpyruvate} + CO_2 + ADP$$

$$\text{Sum: } ATP \longrightarrow ADP + P_i$$

This mechanism is not likely to be operating in *Cl. sporogenes*, however, as this organism lacks a measurable PEP carboxykinase activity, although it does possess PEP carboxylase (N. M. Dixon unpublished results).

EFFECTS OF CO_2 ON THE BIOMASS YIELD OF *Clostridium sporogenes* IN GLUCOSE-LIMITED CHEMOSTAT CULTURE

The effects of p_{CO_2} on the maximum specific growth yield of *Cl. sporogenes* differ between the two growth media. In EAA/FA medium the increase in yield with increasing p_{CO_2} can be attributed to the corresponding decrease in the maintenance requirements. At p_{CO_2} values above 0·5 atm, however, the yield decreased with increasing p_{CO_2}, despite the continued decrease in the maintenance requirements. This, combined with the observed inhibition by CO_2 of the growth rate of this organism in batch cultures (Dixon *et al.* 1987), suggests the occurrence of some type of metabolic uncoupling caused by CO_2. It is as yet unclear, however, as to what in general, at the metabolic level, is (most) limiting to the growth rate of microbial cells in unrestricted batch culture, even between anabolism and catabolism (Kell 1987), and hence it is even less clear how changes in the growth rate limitation may be affected mechanistically. In the attempt to determine whether anabolism or catabolism is most limiting, opposing conclusions have been reached by workers studying batch cultures of the same organism (Andersen & von Meyenburg 1980; Harvey & Koch 1980).

In the present study in MACC medium the biomass yields were independent of p_{CO_2} below 0·5 atm, despite the decreasing maintenance requirements with increasing p_{CO_2}. Above 0·5 atm p_{CO_2}, both the yield values and the maintenance requirements increased with increasing p_{CO_2}, although the growth rates in batch cultures were decreased. Such results are inconsistent with the occurrence of a simple, growth rate-independent metabolic uncoupling.

A non-equilibrium thermodynamic assessment of the efficiency of growth of heterotrophic bacteria indicated that they have in general evolved to permit a maximum metabolic flux of the carbon- and energy-source at the expense of efficiency or yield, so that 'the thermodynamic efficiency of microbial growth is low but optimal for maximal growth rate' (Westerhoff *et al.* 1983; Kell 1987). We have shown that merely by changing the p_{CO_2}, both the maintenance requirements and growth yield of *Cl. sporogenes* are increased, so that one must conclude that the 'efficiency' of growth must also be greater under the more favourable conditions, since a greater yield is being produced from less available free energy. If microbial growth has in fact been optimized for maximum growth rate, an increase in the efficiency of microbial growth (however caused) will cause the system to become suboptimal for maximum growth rate and, hence, the growth rate in batch culture will be reduced. This is exactly what appears to have happened in MACC medium above 0·5 atm p_{CO_2}, although it is clear that no simple relationship between growth rate, maintenance requirement and yield in fact existed in the present case.

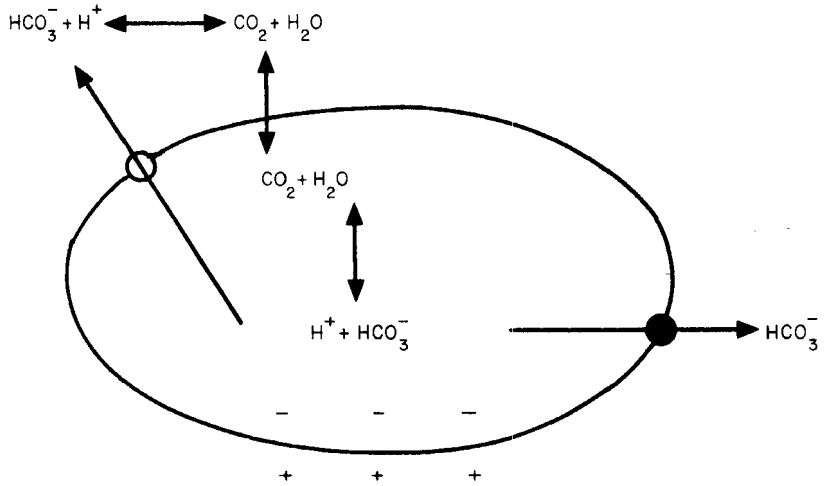

Fig. 4. A possible futile cycle involving CO_2, which freely permeates the biological membrane. Once inside the cell, CO_2 may then react with water to form bicarbonate ions and liberate protons. In an attempt to maintain the internal pH, protons are actively transported from the cell, resulting in the dissipation of energy. The bicarbonate ions may be actively transported from the cell, at a further energetic cost, or may 'leak' from the cell. As the hydration of CO_2 is reversible, the bicarbonate ions may react with a proton to produce water and CO_2, which is able to permeate the membrane, thus completing the cycle. ○, proton pump (ATP hydrolase); ●, HCO_3^- pump or leak.

Metabolic uncoupling was dismissed as the reason for the inhibition of growth rate at high p_{CO_2} in batch culture, due to the lack of inhibition of growth rate in complex media (Dixon *et al.* 1987). It was postulated that the observed inhibition of growth rate was due to the inhibition of particular decarboxylation reactions, which resulted in a lowering in the concentrations of certain essential amino acids controlling the growth rate. Whilst this may still be the cause of the inhibition by high p_{CO_2} of the growth rate in MACC medium, metabolic uncoupling remains a possibility for explaining the inhibition of growth rates by high p_{CO_2} values in EAA/FA medium.

EFFECT OF pH

During the previously reported study of the physiology of *Cl. sporogenes* in chemostat cultures (Lovitt *et al.* 1987c), the chemostats were operated at pH 7·0, as compared with 6·5 in the present report, under a gas phase of 1 atm p_{CO_2}. Comparison of the findings made in these two studies shows that the maximum specific growth yield of this strain, at $p_{CO_2} = 1$ atm, was significantly greater at higher pH, and that this was reflected in the relevant maintenance coefficients. As well as the effects of pH *per se*, these effects could be due to CO_2, HCO_3^- or both. The concentration of dissolved CO_2 (CO_{2aq}), however, is independent of pH, as can be seen from Henry's law (Butler 1982), whereas bicarbonate concentration is a function of pH and p_{CO_2}. The CO_2/HCO_3^- equilibria in the media used for *Cl. sporogenes* have been discussed previously (Dixon *et al.* 1987). Both the yields and the maintenance requirements in EAA/FA glucose-proline medium were significantly lower at a pH of 6·5 than at 7·0, which could be due either to the increased HCO_3^- concentration or to the pH *per se*.

One possibility that does not appear to have been discussed previously is that of metabolic uncoupling via a futile proton cycle (Fig. 4). Carbon dioxide is readily able to permeate membranes whereas HCO_3^- is not (Rubio 1986). Once inside the cell CO_2 will react with water and then dissociate, according to equation (1) (Knoche 1980):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (1)$$

Protons thus liberated within the cell may then be exported, via an ATPase, to maintain the internal pH, at the cost of ATP hydrolysis. The HCO_3^- ion may either leak from the cell via an appropriate porter or be actively exported at further energetic cost to the cell. Bicarbonate in

the external environment may react with a proton, the reverse of equation (1), to yield CO_2 which is able to permeate the cell once again, thus completing the cycle. As the pH increases, so the H^+ ion concentration decreases, and so HCO_3^- present in the external environment will have less chance of reacting with a proton to produce H_2O and CO_2. That this is the case is clear from the shift in the equilibrium in equation (1) from the left to the right as the pH increases (Knoche 1980; Butler 1982). The result of this is that the futile cycle would be less likely to operate as the pH increases, and so less free energy would be wasted. This could simply serve to explain the observed increase in yield at pH 7·0 over that at pH 6·5. The apparent effect of pH on the maintenance requirement may be due to the fact that enzymes catalysing carboxylation reactions are specific for either CO_2 or HCO_3^- (Rubio 1986) and hence may be affected by a shift in the equilibrium between the two species. Overall, our results show that there is no necessary relationship between maximum specific growth yield and maintenance for a given organism, and that a re-evaluation of existing chemostat theory on this point may be opportune.

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References

AHMED, S. & BOOTH, I.R. 1981 The effects of partial and selective reduction in the components of the proton-motive force on lactose uptake in *Escherichia coli*. *Biochemical Journal* **200**, 583–589.

ANDERSEN, K.B. & VON MEYENBURG, K. 1980 Are growth rates of *Escherichia coli* limited by respiration? *Journal of Bacteriology* **144**, 114–123.

BLICKSTAD, E. & MOLIN, G. 1983 The microbial flora of smoked pork loin and frankfurter sausage stored in different gas atmospheres at 4°C. *Journal of Applied Bacteriology* **54**, 46–56.

BUTLER, J.N. 1982 *CO_2 Equilibria and their Applications*. London: Addison Wesley.

BUTTON, D.K. 1985 Kinetics of nutrient-limited transport and microbial growth. *Microbiology Reviews* **49**, 270–297.

CLARK, D.S. & LENTZ, C. P. 1969 The effect of carbon dioxide on the growth of slime producing bacteria on fresh beef. *Canadian Institute of Food Technology Journal* **2**, 72–75

DABES, J.N., FINN, R.K. & WILKE, C.R. 1973 Equations of substrate-limited growth: the case for Blackman kinetics. *Biotechnology and Bioengineering* **15**, 1159–1177.

DE VRIES, W., KAPTEIJN, W.M.C., VAN DER BEEK, E.G. & STOUTHAMER, A.H. 1970 Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. *Journal of General Microbiology* **63**, 333–345.

DIXON, N.M., LOVITT, R.W., KELL, D.B. & MORRIS, J.G. 1987 Effects of pCO_2 on the growth and metabolism of *Clostridium sporogenes* NCIB 8053 in defined media. *Journal of Applied Bacteriology* **63**, 171–182.

EDDY, A.A. 1980 Slip and leak models of gradient-coupled solute transport. *Biochemistry Society Transactions* **8**, 271–273.

FINNE, G. 1982 The effect of carbon dioxide on the bacteriological and chemical properties of foods: a review. In *Proceedings 1st National Conference on Modified and Controlled Atmosphere Packaging of Seafood Products* ed. Martin, R.E. pp. 2–19, San Antonio, Texas: National Fisheries Institute.

FRAZIER, W.C. & WESTHOFF, D.C. 1978 *Food Microbiology* 3rd edn. New York: McGraw-Hill.

GLADSTONE, G.P., FILDES, P. & RICHARDSON, G.M. 1935 CO_2 as an essential factor in the growth of bacteria. *British Journal of Experimental Pathology* **16**, 335–348.

GOTTSCHAL, J.C. & MORRIS, J.G. 1981 Non-production of acetone and butanol by *Clostridium acetobutylicum* during glucose- and ammonium-limitation in continuous culture. *Biotechnology Letters* **3**, 525–530.

HARDER, W., VAN DIJKEN, J.P. & ROELS, J.A. 1981 Utilisation of energy in methylotrophs. In *Microbial Growth on C_1 compounds* ed. Dalton, H. pp. 258–269, London: Heyden.

HARVEY, R.J. & KOCH, A.L. 1980 How partially inhibitory concentrations of chloramphenicol affect the growth of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **18**, 323–337.

HELLINGWERF, K.J., LOLKEMA, J.S., OTTO, R., NEIJSSEL, O.M., STOUTHAMER, A.H., HARDER, W., VAN DAM, K. & WESTERHOFF, H.V. 1982 Energetics of microbial growth: an analysis of the relationship between growth and its mechanistic basis by mosaic non-equilibrium thermodynamics. *FEMS Microbiology Letters* **15**, 7–17.

HILL, T.L. 1977 *Free Energy Transduction in Biology* New York: Academic Press.

JAMES, E.W., KELL, D.B., LOVITT, R.W. & MORRIS, J.G. 1988 Electrosynthesis and electroanalysis using *Clostridium sporogenes*. *Bioelectrochemistry and Bioenergetics*, in press.

JONES, C.W. 1977 Aerobic respiratory systems in bacteria. *Symposia of the Society of General Microbiology* **27**, 23–59.

KACSER, H. & PORTEOUS, J.W. 1987 Control of metabolism: what do we have to measure? *Trends in Biochemical Science* **12**, 5–14.

KEDEM, O. & CAPLAN, S.R. 1965 Degree of coupling and its relation to efficiency of energy conversion. *Transactions of the Faraday Society* **61**, 1897–1911.

KELL, D.B. 1987 Forces, fluxes and control of microbial growth and metabolism. The twelfth Fleming

lecture. *Journal of General Microbiology* **133**, 1651–1665.

KELL, D.B. & WESTERHOFF, H.V. 1985 Catalytic facilitation and membrane bioenergetics. In *Organized Multienzyme Systems: Catalytic Properties* ed. Welch, G.R. pp. 63–139, New York: Academic Press.

KELL, D.B. & WESTERHOFF, H.V. 1986a Towards a rational approach to the optimisation of flux in microbial biotransformations. *Trends in Biotechnology* **4**, 137–142.

KELL, D.B. & WESTERHOFF, H.V. 1986b Metabolic control theory: its role in microbiology and biotechnology. *FEMS Microbiology Reviews* **39**, 305–320.

KNOCHE, W. 1980 Chemical reactions of CO_2 in water. In *Biophysics and Physiology of CO_2* ed. Bauer, C., Gros, G. & Bartell, H. pp. 3–11, Berlin: Springer-Verlag.

KOCH, A.L. 1985 The macroeconomics of bacterial growth. In *Bacteria in their Natural Environment* ed. Fletcher, M.M. & FLOODGATE, G. pp. 1–42, London: Academic Press.

LOVITT, R.W., JAMES, E.W., KELL, D.B. & MORRIS, J.G. 1987a Bioelectrochemical transformations catalysed by *Clostridium sporogenes*. In: *Bioreactors and Biotransformations* ed. Moody, G.W. & Baker, P.B. pp. 265–276, London: Elsevier.

LOVITT, R.W., MORRIS, J.G. & KELL, D.B. 1987b Growth and nutrition of *Clostridium sporogenes* NCIB 8053 in defined media. *Journal of Applied Bacteriology* **62**, 71–80.

LOVITT, R.W., KELL, D.B. & MORRIS, J.G. 1987c The physiology of *Clostridium sporogenes* NCIB 8053 growing in defined media. *Journal of Applied Bacteriology* **62**, 81–92.

MOLIN, G. 1983 Combined carbon dioxide inhibition and oxygen limitation of the growth of *Pseudomonas fragi* 72 in batch and continuous culture. *Journal of General Microbiology* **129**, 2885–2891.

MONTICELLO, D.J., HADIOETOMO, R.S. & COSTILOW, R.N. 1984 Isoleucine synthesis by *Clostridium sporogenes* from propionate or α-methyl-butyrate. *Journal of General Microbiology* **130**, 309–318.

MORRIS, J.G. 1982 Anaerobic fermentations—some new possibilities. *Biochemistry Society Symposia* **48**, 147–172.

MORRIS, J.G. 1986, Anaerobic Metabolism of Glucose. In *Comprehensive Biotechnology* vol. 1 ed. Cooney, C.L. & Humphrey, A.E. pp. 357–378, Oxford: Pergamon Press.

NICOLIS, G. & PRIGOGINE, I. 1977 *Self-organisation in Non-equilibrium Systems* London: Wiley.

NIPKOW, A., SONNLEITNER, B. & FIECHTER, A. 1985, Effect of carbon dioxide on growth of *Zymomonas mobilis* in continuous culture. *Applied Microbiology and Biotechnology* **21**, 287–291.

PIETROBON, D. 1986 A non-linear kinetic model of chemiosmotic energy coupling. *Biochemistry and Bioenergetics* **15**, 193–209.

PIETROBON, D. & CAPLAN, S.R. 1985 Flow-force relationships for a six-state proton pump model: intrinsic uncoupling, kinetic equivalence of input and output forces, and domain of approximate linearity. *Biochemistry* **24**, 5764–5776.

PIETROBON, D., ZORATTI, M., AZZONE, G.F. & CAPLAN, S.R. 1986 Intrinsic uncoupling of mitochondrial proton pumps. 2. Modeling studies. *Biochemistry* **25**, 767–775.

PIRT, S.J. 1975 *Principles of Microbe and Cell Cultivation* Oxford: Blackwell Scientific Publications.

RUBIO, V. 1986 Enzymatic HCO_3^- fixation: a common mechanism for all enzymes involved? *Bioscience Reports* **6**, 335–347.

SENEZ, J.C. 1962 Some considerations on the energetics of bacterial growth. *Bacteriology Reviews* **26**, 95–107.

SPAHL, A., REINECCIUS, G. & TATINI, S. 1981 Storage life of pork chops in CO_2-containing atmospheres. *Journal of Food Protection* **44**, 670–673.

STEPHENS, G.M., HOLT, R.A., GOTTSCHAL, J.C. & MORRIS, J.G. 1985 Studies on the stability of solvent production by *Clostridium acetobutylicum* in continuous cultures. *Journal of Applied Bacteriology* **59**, 597–605.

STOUTHAMER, A.H. 1979 Energetic aspects of the growth of microorganisms. *Symposia of the Society of General Microbiology* **27**, 285–315.

STOUTHAMER, A.H. & BETTENHAUSEN, C.W. 1977 A continuous culture study of an ATP-ase negative mutant of *Escherichia coli*. *Archives of Microbiology* **113**, 185–189.

TEIXEIRA DE MATTOS, M.J., PLOMP, P.J.A.M., NEIJSSEL, O.M. & TEMPEST, D.W. 1984 Influence of metabolic end products on the growth efficiency of *Klebsiella aerogenes* in anaerobic chemostat culture. *Antonie van Leeuwenhoek* **50**, 461–472.

TEMPEST, D.W. 1970 The continuous cultivation of microorganisms. I. Theory of the chemostat. In *Methods in Microbiology vol. 2* ed. Norris, J.R. & Ribbons, D.W. pp. 259–276, London: Academic Press.

TEMPEST, D.W. & NEIJSSEL, O.M. 1984 The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. *Annual Review of Microbiology* **38**, 459–486.

THAUER, R., JUNGERMANN, K. & DECKER, K. 1977 Energy Conservation in chemotrophic anaerobic bacteria. *Bacteriology Reviews* **41**, 100–180.

VAN ANDEL, J.G., ZOUTBERG, G.R., CRABBENDAM, P.M. & BREURE, A.M. 1985 Glucose fermentation by *Clostridium butyricum* grown under a self-generated gas atmosphere in chemostat culture. *Applied Microbiology and Biotechnology* **23**, 21–26.

WANG, M.Y. & OGRYDZIAK, D.M. 1986 Residual effect of storage in an elevated carbon dioxide atmosphere on the microbial flora of rock cod (*Sebastes* spp.). *Applied and Environmental Microbiology* **52**, 727–732.

WESTERHOFF, H.V. & VAN DAM, K. 1979 Irreversible thermodynamic description of energy transduction in biomembranes. *Current Topics in Bioenergetics* **9**, 1–62.

WESTERHOFF, H.V. & VAN DAM, K. 1987 *Thermodynamics and Control of Biological Free-energy Transduction* Oxford: Elsevier.

WESTERHOFF, H.V., HELLINGWERF, K.J. & VAN DAM, K. 1983 Efficiency of microbial growth is low but optimum for maximum growth rate. *Proceedings of the National Academy of Sciences USA* **80**, 305–309.

WESTERHOFF, H.V., LOLKEMA, J.S., OTTO, R. & HELLINGWERF, K.J. 1982 Thermodynamics of growth. Non-equilibrium thermodynamics of bacterial growth: the phenomenological and the mosaic approach. *Biochimica et Biophysica Acta* **683**, 181–220.

WINDASS, J.D., WORSEY, M.J., PIOLI, E.M., PIOLI, D., BARTH, P.T., ATHERTON, K.T., DART, E.C., BYROM, D., POWELL, K. & SENIOR, P.J. 1980 Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus*. *Nature* **287**, 396–401.

ZEIKUS, J.G. 1980 Chemicals and fuel production by anaerobic bacteria. *Annual Review of Microbiology* **34**, 423–464.

ZEIKUS, J.G. 1985 Biology of spore-forming anaerobes. In *The Biology of Industrial Microorganisms* ed. Demain, A.L. & Solomon, N.A. pp. 79–114, California: Benjamin Cummings.

ZORATTI, M., FAVARON, M., PIETROBON, D. & AZZONE, G.F. 1986 Intrinsic uncoupling of mitochondrial proton pumps. 1. Non-ohmic conductance cannot account for the nonlinear dependence of static head respiration on $\Delta\tilde{\mu}_H$. *Biochemistry* **25**, 760–767.