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A Review

The inhibition by CO_2 of the growth and metabolism of micro-organisms

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1. Introduction

It has been known for many years that the growth and metabolism of micro-organisms is accompanied by the uptake and/or evolution of carbon dioxide. Yet, except for the obvious case of autotrophic micro-organisms, the partial pressure of CO_2 (pCO_2) has rarely been considered to be of much quantitative significance to the physiology of the cell. Although there have been a number of reviews on the effects of CO_2 on the requirement for and fixation of CO_2 by micro-organisms, especially autotrophic ones (e.g. Fuchs & Stupperich 1983; Codd 1984; Fuchs 1986), in general little attention appears to have been concentrated on what are in fact a rather widespread set of studies concerning

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CO_2 inhibition. Nonetheless, CO_2 is inhibitory to the growth of a number of micro-organisms, a fact which has enjoyed increasing exploitation in the preservation of foodstuffs from bacterial spoilage. It therefore seemed appropriate to collate and summarize this material, a survey which forms the subject matter of the present review. After an historical introduction, the use of CO_2 as an antimicrobial agent for use in the preparation of potables and consumables is considered. This leads naturally to a review of some of the antimicrobial properties of CO_2 during industrial fermentations. Many of these observations have been of a somewhat empirical nature; thus, finally we consider some of the more molecular or mechanistic studies which have sought to ascertain the means by which CO_2 might be acting to cause microbial growth inhibition. We begin with a brief discussion of the thermodynamics of the interactions of CO_2 and aqueous media.

2. '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 in water, the reaction scheme may be written as (Knoche 1980):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

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 hydration reactions need to be considered:

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

2.1. DISSOLVED CO_2 CONCENTRATION

The concentration of CO_2 in solution ($[CO_2]aq$) is normally expressed by Henry's law (Butler 1982; Ho *et al.* 1987):

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

where K_H = the Henry's law constant (in units of mol/l atm) and pCO_2 = the partial pressure of CO_2 in the gas phase (in atm). For cultures 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:

$$[CO_2]aq = 10^{1.39} \times pCO_2 = 24{\cdot}6 \times pCO_2 .$$

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

2.2. BICARBONATE CONCENTRATION IN A pH-CONTROLLED CULTURE WITH A CONSTANT TEMPERATURE AND GAS PHASE

The equilibrium between CO_2 and HCO_3 is defined by a 'hybrid' equilibrium constant K_1' (Butler 1982) where

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

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^0_1 and the ionic strength I by:

$$pK'_1 = pK^0_1 - 0{\cdot}5f(I) - bI \tag{6}$$

From Davies's equation (Butler 1982),

$$f(I) = [I^{1/2}/(1 + I^{1/2}) - 0{\cdot}21][(298/(T + 273)]^{2/3} \tag{7}$$

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

$$I = 1/2 \sum c_i z_i^2 \tag{8}$$

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

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

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

Therefore from eqn (9) we obtain $pK_{a.1}$, and so from eqn (5)

$$\log_{10}[HCO_3^-] = pH - pK_{a.1} + \log_{10}[CO_2] .$$

A typical value of $pK_{a.1}$ is 6·3 (at zero ionic strength and 35°C, Butler 1982). Thus the addition of CO_2 to an aqueous medium will tend to cause the pH to drop to a-value approximately equal to the pK, depending upon the other buffering constituents in the medium.

3. Historical

Carbon dioxide was known to the ancients. Its presence was perceived by Plenius near volcanoes and mineral springs and it was called by him 'spiritus letales' (Valley & Rettger 1927). John Baptista van Helmont, introducing the word gas, called it 'gas sylvestre' (Valley & Rettger 1927). He knew that this gas could extinguish a flame and cause suffocation in animals. It is possible that these, now well-known, properties caused the efforts of the earlier workers to be for the most part directed towards a study of the inhibitory action of CO_2 on the growth of bacteria. Amongst the earliest work concerning the influence of CO_2 on bacteria was that of Pasteur & Joubert (1877), who reported that *Bacillus anthracis* was killed by CO_2. No mention was made, however, of the media used, the length of the exposure to CO_2, or the concentration of CO_2 required for a fatal effect. Further research showed that exposure to an almost pure atmosphere of CO_2 for 5–8 h did not in fact kill *B. anthracis*, nor even alter its pathogenicity (Szpilman 1880), although results from around this time were often contradictory (e.g. d'Arsonval & Charrin 1893; Sabrazès & Bazin 1893). *Bacterium typhosum* (*Salmonella typhi*) and *B. anthracis* were found by Schaffer & Freudenreich (1891) to be unaffected in broth cultures under 7 atm pressure of CO_2. (N.B. 1 atm pressure = 14.696 lbf/in^2 = 101 325 Pa = 760 mm Hg; despite the fact that atm are not SI units, the widespread usage of this unit, and the intuitive feel for what it represents, leads to the retention of its usage herein.) It was also reported that CO_2 does not kill bacteria, but inhibits motility, whereas small amounts of CO_2 stimulated motility (Grossman & Mayerhausen 1877). In contrast to this, CO_2 was found to be toxic to *Meningococcus* spp. and to exert a rapid toxic effect upon *Spirochaeta pallida* (Shaw-Mackenzie 1918). It was mentioned in the same paper that CO_2 gas could be used beneficially in the treatment of open wounds and ulcerations, as well as by rectal introduction in cases of dysentery! That the inhibitory effect of CO_2 is organism-dependent appears first to have been shown by Buchner (1885), in that CO_2 inhibited Koch's Vibrio, but growth was obtained with Ficth's Aethylbacillus, the Napel cholera bacillus and *Salm. typhi*.

4. Treatment of water with CO_2

About the turn of the century, carbonation was being investigated as a treatment for the improvement of the quality of water, milk and milk products. In fact, the carbonation of beverages is today one of the largest applications of CO_2 (Reichert 1982). Reduction in bacterial numbers in Munich city water was obtained in the laboratory by carbonating the water at atmospheric pressure, but sterilization was not reported (Leone 1886). That it was CO_2, and not the lack of oxygen, that caused the decrease in bacterial numbers had been demonstrated earlier (Leone 1885). Artifically carbonated

Table 1. The effect of pH and CO_2 on the growth of micro-organisms (Hays *et al.* 1959)

	Carbon dioxide				Nitrogen		
	Controls		Samples		Controls		Samples
Organism	pH	Growth	pH	Growth	pH	Growth	Growth
Klebsiella aerogenes	6·7	+ +	5·6	−	5·6	+ +	+ +
Clostridium botulinum	6·9	+ +	5·4	±	5·4	+ +	+
Clostridium butyricum	5·5	+ +	5·0	±	5·0	+ +	+ +
Clostridium sporogenes	6·9	+ +	5·4	±	5·4	+ +	+ +
Staphylococcus aureus	6·7	+ +	5·6	±	5·6	+ +	+ +
Saccharomyces cerevisiae	5·5	+ +	5·0	−	5·0	+ +	+ +
Pink torula	5·5	+ +	5·0	−	5·0	+ +	+ +
Aspergillus niger	5·5	+ + +	5·0	±	5·0	+ + +	+ +

Controls were not gassed whereas the samples were pressurized to approximately 6 atm of either CO_2 or N_2. The pH of the CO_2 controls was the 'normal' pH of the medium, the pH of the CO_2 samples was the pH after the addition of the CO_2 and the pH for the N_2 controls and samples was adjusted to match that for the CO_2 samples. The degree of growth was defined as follows: + + +, luxuriant mould growth with aerial hyphae; + +, marked increase in colony count over initial count or moderate mould growth; +, definite growth or slight to moderate increase in colony count; ±, colony count remained approximately equal to the initial count; −, slight decrease in colony count from the initial count or no evidence of mould growth.

waters, distilled water and Berlin city water were used to demonstrate that carbonation removed the danger from cholera, but that there was still danger from typhoid fever, since *Salm. typhi* remained viable in carbonated waters for from 5 to 7 d (Hochstetter 1887), and in some cases growth continued unhindered (Fränkel 1889; Frankland 1896). *Vibrio cholerae*, *B. anthracis*, *Staphylococcus aureus*, *Staph. albus*, *Salm. typhi* and '*B. lepisepticum*' were not affected by the concentration of CO_2 naturally dissolved (from the atmosphere) in water at 15°C, and only *V. cholerae* and *B. anthracis* were affected when the water was saturated with CO_2 (Scala & Sanfelice 1891). When *Pseudomonas fluorescens* var. *liquefaciens*, *Escherichia coli* var. *communis*, *Staph. aureus*, *Salm. typhi* and *V. cholerae* were inoculated into carbonated waters, killing or inhibition could be effected, the extent depending upon the organism and intensifying with time (Slater 1893). Reduction of pathogenic organisms, but not sterilization, could be obtained in carbonated beverages (Young & Sherwood 1911), the bactericidal action of CO_2 being assumed to be due to the increased hydrogen ion concentration of the solution (Koser & Skinner 1922; Valley & Rettger 1927). Later experimental data (Becker 1933; Hays *et al.* 1959), however, indicated that CO_2 *per se*, rather than its effect on pH, was the cause of the inhibitory effect (Table 1).

The decline in cell numbers of *E. coli* and *Salm. typhi* in artificially inoculated, carbonated beverages, was apparently proportional to the pressure and storage time (Donald *et al.* 1924), and apparent sterilization was achieved in only a few instances. When *E. coli* was subjected to 350 atm of CO_2 the number of bacteria was reduced by approximately one half in 20 min (Swearinger & Lewis 1933). More recently the aerobic growth rate of *E. coli* in fed-batch cultures was found not to be inhibited by 0·2 atm pCO_2, measured in the effluent gas, but 17% and 21% inhibition of growth rate was observed respectively at $pCO_2 = 0{\cdot}64$ atm in complex medium and $pCO_2 = 0{\cdot}62$ atm in defined medium (Mori *et al.* 1983). In the same study, inhibition of the growth rate of *Candida brassicae* was not observed until the pCO_2 in the effluent gas was 0·61 atm. During anaerobic growth of *E. coli* the optimum growth rate was at a dissolved CO_2 concentration of 1·3 mmol/l ($\approx 0{\cdot}05$ atm pCO_2 in the gas phase), above which the growth rate was inhibited (Lacoursiere *et al.* 1986). The perhaps not unexpected conclusion that was drawn from this, was that the maximum growth rate of *E. coli* occurs at CO_2 concentrations close to that found in the mammalian gut, where *E. coli* naturally resides.

The spoilage microflora of carbonated beverages are dominated by yeasts, and the carbonation tolerance of some of these was recently investigated (Ison & Gutteridge 1987). The most tolerant yeasts of those studied were *Dekkera anomala* and those of the genus *Brettanomyces*, a finding which

is in accordance with *Brettanomyces* spp. being the sole spoilage micro-organisms of soft drinks with high carbonation levels (Ison & Gutteridge 1987).

5. Treatment of dairy products with CO_2

The bacterial count of milk could be reduced by 50 atm of CO_2, but sterilization was still never obtained (Hoffman 1906). Milk kept under 10 atm of CO_2 remained in good condition for 72 h, whereas untreated milk curdled in 24 h. Increased pressures of CO_2 delayed lactic fermentation but no noticeable effect of CO_2 was observed at atmospheric pressure (Van Slyke & Bosworth 1907). Such findings led to a number of studies on the possible antibacterial effect of CO_2 in other dairy products.

Experiments on the effect of CO_2 on butter indicated that carbonation could not be relied upon as a means of destroying bacteria present in cream and rendering such cream, or the butter made from it, safe for human consumption (Hunziker 1924). Similarly, carbonation could not be relied upon to improve the keeping quality or to prevent flavour deterioration of the resulting butter. If it was made from unpasteurized cream, carbonated butter developed the usual bacterial flavour defects (Hunziker 1924). It was concluded that if any appreciable benefit was to be obtained from the carbonation of butter, it would be necessary to store the butter in a CO_2 atmosphere, although in time butter stored in this way also developed undesirable flavours (Prucha *et al.* 1925). Carbonation of the cream, or of the butter during churning, did not result in any benefit to justify the use of CO_2 in this way (Prucha *et al.* 1925).

Carbonation of ice-cream had no appreciable effect on the bacteria within the ice-cream (Prucha *et al.* 1922; Rettger *et al.* 1922). It was demonstrated that CO_2 at atmospheric pressure had no bacteriostatic or bacteriocidal effect on organisms originally present in the ice-cream, or on *Streptococcus lactis*, *E. coli* or *B. cereus* added to it (Valley & Rettger 1927). No marked differences in viability could be discerned, and the slight variations favoured the aerated products and not the CO_2-treated products. In any event, after 7 weeks there was no difference in the CO_2 content of carbonated and and uncarbonated ice-cream due to the egress of the CO_2 by diffusion. Carbonation did not enhance the keeping quality of milk, nor prevent rapid bacterial proliferation of the pre-existing flora, nor of *Strep. lactis*, *E. coli*, *Salm. typhi* nor *Salm. hirschfeldii* inoculated into the milk (Valley & Rettger 1927).

Thus, although bacterial activities could be inhibited in the case of carbonated beverages, sterilization of water, milk or milk products was not possible simply by carbonation, and this was the general conclusion of a number of reports reviewed by Valley (1928). The carbonation of beverages was successful because the beverages were kept in air-tight containers under pressure. In order to inhibit the bacterial activities in dairy products higher pressures than those used in carbonated beverages, for which 4·8 atm were recommended (Donald *et al.* 1924), would be required (Prucha *et al.* 1925). The pH of carbonated beverages tends to be somewhat lower than that of milk, and since conditions causing inhibition are often synergistic, this would have also contributed to the success with carbonated beverages.

More recently, when milk was stirred under a headspace of 1 atm pCO_2 the pH fell to 6·0 and the CO_2 content increased to about 30 mmol/l. When this milk was subsequently held at 4 or 7°C there was a pronounced inhibitory effect on the growth of psychrotrophs, as compared with controls in air (Law & Mabbitt 1983). If 10^6 bacteria/ml was taken to be the point when degradative changes in the milk become unacceptable, then the CO_2 treatment extended the possible storage time by about 3 d (at 4°C) or 2 d (at 7°C) for 'poor' quality milk and even longer for 'good' quality milk. The CO_2 could easily be removed before pasteurization, although this was not necessary if the milk was for cheese or yoghurt.

High concentrations of CO_2, combined with low oxygen concentrations, play an important role in the ripening of blue cheese. *Penicillium roqueforti* is mainly responsible for the ripening of blue cheese. As blue cheese ripens, its oxygen content decreases rapidly, and the CO_2 content rises. These conditions are unfavourable to all moulds, but they tend to affect *Pen. roqueforti* to a lesser extent than other species likely to be present and able to grow at the salt concentration found in blue cheese

(Thom & Currie 1913; Foster *et al.* 1958). It has been suggested that piercing the cheese stimulates the growth of this mould by allowing CO_2 to escape (Golding 1937).

6. Preservation of meat and fish by CO_2

Partly in view of the growth-inhibitory effects of CO_2 that had been observed in some cases above, attempts, which began at approximately the same time as the early work on the carbonation of water, were also made to preserve meat and fruit in an atmosphere of CO_2. Beef could be so preserved for 18 d in hot summer weather with daytime temperatures up to 32°C, whilst mutton showed spoilage in a very short time (Kolbe 1882). It is not known what the contribution of the pH was to this effect. Pork and lamb which went bad in 10 d in air at 4·5–7·2°C remained free from spoilage for 3 weeks when stored in CO_2 (Killeffer 1930). Partial pressures of CO_2 in the atmosphere suppressed mould growth on meat (Moran *et al.* 1932) and the growth of *Pseudomonas* and *Achromobacter* spp. in nutrient broth at 0 and 4°C (Haines 1933). Further work showed inhibition of the growth of such organisms on ox-muscle at -1°C in 0·1 atm pCO_2 (Empey & Scott 1939). The first practical use of modified atmospheres containing elevated levels of CO_2 as a preservative in the handling of fresh meat was in the shipment of whole chilled beef carcasses from Australia and New Zealand to Britain in the 1930s. By 1938, 26% of the beef from Australia and 60% of that from New Zealand was being shipped under CO_2 atmospheres (Lawrie 1974). The ability of high concentrations of CO_2 ($>0·1$ atm) to retard the growth of the Gram-negative spoilage flora of meat, poultry and fish, and in this way to prolong the shelf-life, is now well documented (Table 2) and modified atmosphere packaging has become the subject of over 4000 scientific papers in the past half decade (Lioutas 1988). Commercially the beneficial effects of CO_2 on the shelf-life of meat are used most during prolonged storage in refrigerated bulk containers (Taylor 1971; Dainty *et al.* 1983), as well as for wholesale and retail packages (Molin *et al.* 1983; Lioutas 1988; Young *et al.* 1988). Gas-packaging has also been applied commercially for poultry meat (Timmons 1976; Mead 1983) on an extensive scale in the US (Hotchkiss 1988). In addition to the use of CO_2 in gas-packaging, CO_2 pellets have been used to chill poultry (Thomson & Risse 1971), lamb carcasses and beef wholesale cuts during shipment from the packers to the retail trade (Smith *et al.* 1974).

6.1. INHIBITION OF THE GROWTH AND METABOLISM OF FOOD-SPOILAGE MICRO-ORGANISMS

In general, the rate of bacterial multiplication decreases, and the length of the lag phase increases, with increasing levels of CO_2 (e.g. Tomkins 1932; Haines 1933) and Gram-positive species are more resistant to the effects of CO_2 than are Gram-negative species (Sutherland *et al.* 1977; Silliker &

Table 2. Examples of the use of CO_2 for the extension of the shelf-life of meat and fish

Foodstuff	Selected references
Beef	Clark & Lentz 1972, 1973; Partman *et al.* 1975; Silliker *et al.* 1977; Sutherland *et al.* 1977; Christopher *et al.* 1979a
Pork	Huffman 1974; Silliker *et al.* 1977; Christopher *et al.* 1979b, 1980; Enfors *et al.* 1980
Cured meats	
Smoked pork	Blickstad & Molin 1983
Frankfurters	Ogilvy & Ayres 1951b, 1953; Blickstad & Molin 1983
Poultry	Ogilvy & Ayres 1951a; Gardner *et al.* 1977; Sander & Soo 1978; Mead 1983
Fish	Coyne 1932; Stansby & Griffiths 1935; Banks *et al.* 1980
Cod	Coyne 1933a; Jensen *et al.* 1981
Herring	Molin *et al.* 1983b
Crayfish	Wang & Brown 1983
Salmon	Stier *et al.* 1981; Fey & Regenstein 1982

The use of CO_2 for the prevention of food-spoilage by micro-organisms and the possible mechanisms of action are discussed in the text.

Wolfe 1980; Stier *et al.* 1981). These effects vary with the concentration of CO_2, incubation temperature, organism and water activity of the medium (Wodzinski & Frazier 1961). Both aerobic respiration and growth rate were inhibited by CO_2 in fluorescent and non-fluorescent *Pseudomonas* spp., *Alteromonas putrefaciens* and *Yersinia enterolitica*, although the inhibition was incomplete (Gill & Tan 1980). The inhibition pattern was apparently biphasic for each organism, such that above a certain pCO_2 a further increase in pCO_2 had a minor inhibitory effect, as in an earlier report on *Ps. fluorescens* (Gill & Tan 1979). In contrast to such organisms, *Acinetobacter* (Gill & Tan 1980), *Ps. aeruginosa* (King & Nagel 1975), *Ps. fragi*, *B. cereus* and *Strep. cremoris* (Enfors & Molin 1980) all displayed a degree of inhibition by CO_2 that was more or less proportional to the CO_2 partial pressure over the entire CO_2 pressure range tested. The relative inhibitory effect of CO_2 (RI) was defined as:

$$RI = [(r_c - r_{CO_2})/r_c] \times 100 \quad (10)$$

where r_c = the growth rate of the control culture, and r_{CO_2} = the growth rate of the CO_2-inhibited culture (Enfors & Molin 1981). As the temperature decreased, the RI for *Ps. fragi* and *B. cereus* increased (Enfors & Molin 1981), as was the case with most of the examples above. The ratio of the RI : CO_2 solubility at the corresponding temperature was approximately constant, and independent of temperature. Hence the increased inhibition by CO_2 at lower temperatures was explained as being due to increased CO_2 solubility, as opposed to increased susceptibility to CO_2. Since the initial microbial activity on muscle foods is located at the surface, however, it is a gas–solid interface that is being dealt with rather than a population dispersed in a liquid medium (Finne 1982a). The temperature effect may not therefore display the same characteristics for such an interfacial phenomenon as for a liquid medium. Results from a number of other authors were treated in a similar fashion: the RI : concentration of dissolved CO_2 was plotted against temperature and similar conclusions were drawn (Ogrydziak & Brown 1982), although in this case a number of the figures were plots of only two or three points.

The inhibitory effect of CO_2 on *Pseudomonas* spp. has been frequently studied [e.g. Clark & Lentz 1969 (several strains), King & Nagel 1975 (*Ps. aeruginosa*), Gill & Tan 1979 (*Ps. fluorescens*), 1980 (fluorescent and non-fluorescent strains), Enfors & Molin 1980 (*Ps. fragi*)] since this genus contains some of the major spoilage organisms of proteinaceous foodstuffs. Many of the results differ, however, with respect to the shape of the curve of growth rate versus CO_2 concentration. Explanations could be offered in terms of the effect of growth substrate or the inherent genetic differences between different species or strains. Some of the discrepancies may also be blamed on the application, in some cases, of fairly crude systems to measure the growth rate. All of the above experiments were carried out in batch cultures, where it was observed that the (maximum) specific growth rate of *Ps. fragi* decreased as the pCO_2 in the effluent gas was increased. However, results from continuous cultures revealed a shoulder (between 0·035 and 0·12 atm CO_2) in the curve of maximum specific growth rate (calculated from critical dilution rates) vs. pCO_2 that was not seen in the batch growth rate determinations (Molin 1983a). In oxygen-limited cultures the growth restricting effect of CO_2 was much more severe (Molin 1983a).

Organisms which were CO_2-sensitive when grown aerobically were unaffected by CO_2 when grown anaerobically, as were *Enterobacter* spp. and *Brochothrix thermosphacta* (Gill & Tan 1980), suggesting that the sites of CO_2 inhibition during aerobic growth are different from those during anaerobic growth.

In general the partial pressures used in determining the degree of inhibition by CO_2 on the growth of the food-spoilage bacteria were between 0 and 1 atm. When hyperbaric CO_2 pressures were used it was found for instance that the time required to reach a given aerobic (*sic*) count was three times longer in 5 atm pure CO_2 than in 1 atm CO_2, and 15 times longer in 5 atm CO_2 than in air (Blickstad *et al.* 1981). These high partial pressures of CO_2 have a considerable effect in prolonging the shelf-life of unsterilized foodstuffs, by virtue of the fact that they select the microflora in favour of *Lactobacillus* spp. which are non-pathogenic (Shaw & Nichol 1969; Roth & Clark 1975) (these are the organisms that are among the least sensitive to high values of pCO_2) and by reducing the growth rate of even these *Lactobacillus* spp. (Ogilvy & Ayres 1953; Blickstad *et al.* 1981; Johnson *et al.* 1982;

Blickstad & Molin 1983). Hence the preserving action of CO_2 is not so much due to the control of the total microbial population as to a restriction of the types of organisms which most rapidly cause deterioration. The selection of the *Lactobacillus* spp. is viewed to have an added benefit, as many lactic bacteria are known to exert an antagonistic effect against other bacteria (Price & Lee 1970; Schroder *et al.* 1980). Nisin, of course, is well known as an antibiotic secreted by certain lactic acid bacteria (Fowler *et al.* 1975; Hurst 1981).

6.2. CO_2-RESISTANCE OF FOOD-RELATED BACTERIA

A number of attempts have been made to group different types of food-related bacteria with regard to their CO_2-resistance (e.g. Coyne 1933b; Sutherland *et al.* 1977; Gill & Tan 1980), but the methods used to evaluate growth rates were relatively crude. A more recent study (Molin 1983b) determined the resistance to CO_2 of a number of such food-related bacteria. Of the organisms studied, the relative growth-inhibitory effect of 1 atm pCO_2, as compared with the growth rate in air, was the highest (>75%) for *B. cereus, Brochothrix thermosphacta* and *Aeromonas hydrophila*, and lowest (29–53%) for *E. coli, Strep. faecalis* and *Lactobacillus* spp. Under nitrogen the relative inhibitory effect of 1 atm pCO_2 on growth rate was lower than for aerobic growth; under anaerobic conditions it was highest for *B. cereus, A. hydrophila* and *Y. frederiksenii* (52–67%), and lowest for *Y. enterolitica, Broch. thermosphacta* and *Lactobacillus* spp. (8–26%). In 1 atm pCO_2 *Strep. faecalis, Citrobacter freundii* and *E. coli* had the highest maximum specific growth rates, and *Broch. thermosphacta, B. cereus* and *Staph. aureus* the lowest.

6.3. CO_2 IN THE PREVENTION OF FOOD SPOILAGE BY CLOSTRIDIA

Although 1 atm pCO_2 inhibited the germination of *B. cereus* spores, the same conditions stimulated the germination of spores of *Clostridium sporogenes* and *Cl. perfringens* (Enfors & Molin 1978a). Germination of *Cl. sporogenes* spores was inhibited slightly at 4 atm and almost completely at 10 atm, whereas germination of *Cl. perfringens* was slightly stimulated at 4 atm, unaffected at 10 atm and stopped at 25 atm of pure CO_2 (Enfors & Molin 1978a). In addition to decreasing the rate of spore germination, CO_2 has been shown to be lethal to certain clostridia (Hays *et al.* 1959). The number of spores of *Cl. butyricum, Cl. botulinum* and *Cl. sporogenes* are decreased after 42 d in the presence of 7·1 atm of CO_2.

Toxin production by *Cl. botulinum* was delayed in 1 atm pCO_2 when compared with that in 1 atm pN_2 at atmospheric pressure (Doyle 1983). Increasing the pressure of CO_2 further delayed the onset of toxin production, although production was not totally inhibited at 8·8 atm of CO_2. At high partial pressures, CO_2 was lethal to *Cl. botulinum*, the rate of decrease of colony-forming units being dependent both upon the CO_2 pressure and the length of exposure to it. However, 8 atm pCO_2 did not serve as a fully antibotulinal agent (Doyle 1983).

The effect of CO_2-enriched atmospheres on the behaviour of food-poisoning organisms in poultry products has received comparatively little attention. There was no significant difference between the effect of CO_2 and N_2 atmospheres, at 43°C, on the growth of eight strains of *Cl. perfringens*, although there was a slightly increased lag phase with two of the strains under CO_2 (Parekh & Solberg 1970). For one strain of *Cl. perfringens* incubated at 20°C the lag phase was 31 h under 1 atm pN_2 and 68 h under 1 atm pCO_2 (Mead 1983), which suggests that clostridial growth could at least be delayed by the presence of CO_2 under marginal growth-temperature conditions. In relation to chicken breast portions 1 atm pCO_2 markedly reduced the growth rate of the micro-organisms present (Gibbs & Patterson 1977).

6.4. PROBLEMS ASSOCIATED WITH THE PRESERVATION OF MEAT BY CO_2-CONTAINING ATMOSPHERES

The ability of modified atmospheres to preserve non-sterile foodstuffs has been shown to increase with increasing pCO_2. However, several workers indicate that the concentration of CO_2 which may

be used for meat storage is limited by the surface browning that occurs when the pCO_2 exceeds 0·2 atm (Pohja *et al.* 1967; Ledward 1970; Silliker *et al.* 1977), although others do not agree (Seidman *et al.* 1979). The browning was thought to result from metmyoglobin formation (Brown & Mebine 1969; Ledward 1970; Adams & Huffman 1972; Silliker *et al.* 1977) since surface browning does not occur in low-myoglobin-content foods, including seafoods (Coyne 1933a; Stansby & Griffiths 1935). This is of primary importance to the consumers who assume that an acceptable colour of the lean indicates freshness; for example the colour of pork chops changed from greyish pink to a less desirable brownish or tan colour as storage time increased (Adams & Huffman 1972). However, the odour of pork chops became objectionable before they were rejected because of appearance (Spahl *et al.* 1981)!

One way in which the development of the detrimental effect of CO_2 on the colour of meat may be circumvented by the addition of CO to the atmosphere, or by pretreatment with CO before the meat is placed in the CO_2-enriched atmosphere for storage (Silliker & Wolfe 1980). Because of safety considerations, however, CO in modified atmospheres has not yet been approved by the regulatory agencies for commercial use in the packaging of fresh foods (Finne 1982a). In most fresh red meat applications a mixture of gases is used and a number of combinations have been suggested e.g. 80% O_2/20% CO_2 (Georgala & Davidson 1970), 85–90% O_2/10–15% CO_2 (Clark & Lentz 1973), 75% CO_2/15% N_2/10% O_2 (Hotchkiss 1988). These gases and their concentrations should be tailored to the individual product, but for nearly all products this will be some combination of CO_2, N_2 and O_2. Nitrogen serves as a filler to keep the package from collapsing as CO_2 dissolves into the product (Hotchkiss 1988). Oxygen is used to maintain the bloomed colour of the meat (Young *et al.* 1988) and to inhibit the growth of anaerobic pathogens, but in many cases does not extend the shelf life (Hotchkiss 1988); CO_2 is added to inhibit the respiration of the product (Kadar 1980) in addition to the growth and metabolism of micro-organisms.

6.5. PRESERVATION OF SEAFOOD BY CO_2-CONTAINING ATMOSPHERES

Carbon dioxide has also been found to be effective in inhibiting the growth of the normal spoilage microbes of fish (Coyne 1932, 1933a, Finne 1982b; Parkin & Brown 1982; Tomlins *et al.* 1982), and modified atmosphere packaging, containing CO_2, is presently used in the seafood industry for bulk shipments (Banks *et al.* 1980; Bell 1980; Beals 1982; Wilhelm 1982). CO_2-enriched atmospheres have also been effective in retarding microbial growth during refrigerated storage of retail packaged seafood products (Finne 1982c; Lannelongue *et al.* 1982a).

As most of our seafoods are still harvested from the wild, the microbial flora is usually diverse, with the majority belonging to the genera *Pseudomonas, Acinetobacter, Moraxella, Flavobacterium-Cytophaga* and *Arthrobacter*, and will of course be affected by the environment from which the seafood is harvested (i.e. regional and seasonal differences), physiological condition of the animal, time between harvest and arrival at the docks, onboard handling, etc. (Lee 1982).

Concern has been expressed, in terms of controlled and modified-atmosphere storage (Clark & Takacs 1980), with regard to the psychrotrophic *Y. enterolitica*, and *Campylobacter fetus* ss. *jejuni*, since CO_2-enrichment is in fact used for selective isolation of these organisms (Skirrow 1977). However, seafood has not been implicated in infections caused by these organisms (Lee 1982).

Further concern with modified atmosphere storage of seafoods was related to the possibility of toxin production by *Cl. botulinum*, especially the psychrotrophic type E, before the fish spoils (Stier *et al.* 1981; Eklund 1982a, 1982b; Post *et al.* 1985), as up to 0·9 atm CO_2 had no effect on growth or toxin production (Silliker & Wolfe 1980, Lee 1982). To overcome this problem, fish could be stored refrigerated in CO_2 up to the period of retail display and then transferred to air (Bell 1982). For pork and beef there was a residual inhibitory effect of the CO_2-containing atmosphere after transfer to air (e.g. Silliker *et al.* 1977; Enfors *et al.* 1979; Silliker & Wolfe 1980; Wolfe 1980; Spahl *et al.* 1981), an effect that was also observed with poultry (Ogilvy & Ayres 1951a; Bailey *et al.* 1979; Silliker & Wolfe 1980).

While it may not be possible to prove that the potential contamination of fish, by *Cl. botulinum* toxin, stored in CO_2-rich atmospheres does not exist, there is evidence that it may not present a significant risk provided proper sanitation and temperature controls are employed (Daniels *et al.*

1985). The potential for contamination could also be reduced by maintaining the temperature below 3·3°C (Schmidt *et al.* 1961). Clostridial growth and toxin production has been detected in fish stored in vacuum- and CO_2-packages, but, in all instances, the fish was spoiled beyond hope of human consumption (Banner 1978). Until safety from botulism can be demonstrated, the use of packaging involving low oxygen concentrations cannot be recommended for retail use (Wilhelm 1982). However, even if the toxin is formed, normal cooking operations will inactivate it (Licciardello *et al.* 1967).

Although CO_2 was effective in prolonging the shelf-life of fresh finfish [the most effective combinations of gas were 1 atm CO_2 or 0·4 atm CO_2/0·6 atm N_2 (Lannelongue *et al.* 1982b)], the rate of microbial growth in the finfish after the removal of the CO_2 paralleled the rate of growth in fish stored without CO_2 (Banks *et al.* 1980). A residual inhibitory effect on microbial growth due to storage in 0·8 atm CO_2 was demonstrated for cod fillets stored in a modified atmosphere and then transferred to air at 4°C (Wang & Ogrydziak 1986). It was suggested that this residual effect was not due to retention of CO_2 at the surface of the fillets but was probably due to the microbial ecology of the system. After 7 d storage, with CO_2, *Lactobacillus* spp. and *Alteromonas* spp. were predominant, and 6 d after transfer to air *Pseudomonas* spp. were again dominant (Wang & Ogrydziak 1986).

Pseudomonads, which are among the major spoilage organisms for seafood stored refrigerated in air (Shaw & Shewan 1968), were inhibited during storage in CO_2 (Enfors & Molin 1980; Gill & Tan 1979). One possible problem during the storage of non-sterile foodstuffs in elevated CO_2 atmospheres is that the spoilage flora may genetically adapt to high levels of CO_2, as appeared to be the case with *Pseudomonas* spp.-like strains isolated from rock cod (Johnson & Ogrydziak 1984). On rock cod fillets stored in 0·8 atm CO_2 at 4°C, *Lactobacillus* spp. and an *Aeromonas*-like organism became the predominant organisms, whilst *V. parahaemolyticus*, *Staph. aureus* or *Cl. botulinum* type E were not isolated from fresh or modified atmosphere stored fillets (Mokhele *et al.* 1983). Similar results were obtained with herring fillets (Molin *et al.* 1983). The initial microflora of fresh herring was dominated by coryneforms, *Flavobacterium* spp., *Moraxella*-like organisms and *Pseudomonas* spp. In air the spoilage flora was dominated by *Pseudomonas* spp. and *Moraxella*-like organisms, whereas homofermentative *Lactobacillus* spp. were the only organisms isolated from herring fillets stored in 1 atm CO_2 at 2°C (Molin *et al.* 1983).

In addition to retarding microbial growth on seafoods, CO_2-enriched atmospheres are also effective in reducing the rate of amine production within the seafood itself (Brown *et al.* 1980, Parkin *et al.* 1981; Johnson *et al.* 1982; Watts & Brown 1982). Whether this is a direct effect on the redox activities of the components of the respiratory chain leading to trimethylamine *N*-oxide is not apparently known.

7. Preservation of fruits and vegetables by CO_2

Much of the value of CO_2 treatment of fruits is due to the delay of their rotting by fungi but this is not completely attributable to a direct influence on the fungal organisms, since pCO_2 influences the physiological conditions of the host tissue itself (Smith 1963). CO_2 is not in general as important a factor in reducing the amount of fungal growth as is lowering the temperature (Brown 1922). Partial pressures of CO_2 in the range of 0·2–0·5 atm nonetheless provided a strong check to fungal growth at all temperatures (Brown 1922; Brooks *et al.* 1932). In comparison with air, 1 atm CO plus 0·5 atm CO_2, plus 0·23 atm 0_2 gave an 80–90% reduction of rot development in strawberries (from *Botrytis cinerea*), apples (*Pen. expansum*), lemons (*Whetzelinia sclerotiorum*) and oranges (*Pen. italicum* and *Pen. digitatum*) (El-Goorani & Sommer 1979). The effectiveness of the CO_2 treatment depends, of course, upon the amount of nutrient available to the fungus, and with greater retardation of fungal growth (due to CO_2) at lower temperatures (Brown 1922; Moran *et al.* 1932). The early literature concerning the effects of CO_2, including the prevention of fungal growth, on the storage of a variety of fruits and vegetables has been reviewed by Smith (1963). The inhibitory effects of CO_2 have also been exploited in the Böhi process for the preservation of grape juice (Jenny 1952). More recently the use of modified atmospheres, containing CO_2, have been limited to the international movement of selected vegetables and fruits and for large-scale domestic transport of apples, pears, citrus fruits and

cabbage (Brecht 1980). Zagory & Kader (1988) reviewed the use of modified atmosphere packaging for the storage of fresh produce.

8. Control of the production of fermented foods and beverages using CO_2

It has been indicated above that under appropriate conditions CO_2 can preserve foods by virtue of its inhibitory influence upon certain food-spoilage organisms. However, the production of many foodstuffs of course actually *relies upon* fermentation processes (Haas 1976, Rose 1981, 1982; Erichsen 1983; Marshall & Law 1984; Campbell-Platt 1987). Thus, CO_2 may also be used as a means by which to control or affect the properties of foods produced by fermentation. An example of this is the use of exogenous CO_2 as a controlling agent in the production of alcoholic beverages by the brewing industry (Hoggan 1980). The final concentration of many flavour compounds and esters may be decreased by increasing the CO_2 pressure during fermentation (Drost 1977). The fermentation rate, the rate and extent of yeast growth, and the final concentration of fusel oils are all decreased by increasing CO_2 pressure, whilst the final pH is increased (Kunkee & Ough 1966, Jones & Greenfield 1982; Arcay-Ledezma & Slaughter 1984).

Fermentation of malt extract medium by *Saccharomyces cerevisiae* under a CO_2 pressure of 1·97 atm resulted in a changed pattern of absorption of amino acids in the first 4 h, with a general excretion of amino acids thereafter (Slaughter *et al.* 1987). Growth of *Sacch. cerevisiae* was almost completely stopped by 2·7 atm of CO_2, but no changes in growth rate were observed in 2·7 atm of N_2 (Norton & Krauss 1972). At much higher pressures, of some 41 atm, inhibition of the growth rate of many organisms occurs, as demonstrated with *Strep. faecalis* at high hydrostatic pressures and high partial pressure of inert gases, including nitrogen (Fenn & Marquis 1968). Inhibition of cell division and of the production of new buds by *Sacch. cerevisiae* was caused by CO_2 when it is produced endogenously or added. In contrast, metabolic production of CO_2 was unaffected by endogenously-produced pressures which inhibited the cell division of this organism. Although cell division was inhibited, doubling of the DNA content of the cells still occurred, indicating that the inhibition of cell division was not due to the inhibition of DNA replication (Norton & Krauss 1972). Despite the increase in DNA content of the cells the content of RNA and protein per cell decreased (Lumsden *et al.* 1987). After one hour at elevated CO_2 pressures the mean cell volume of *Sacch. cerevisiae* had increased, whereas the mean cell volume of *Schizosaccharomyces pombe* had decreased, suggesting that the influence of CO_2 upon cell characteristics may be associated with a change in cell volume (Lumsden *et al.* 1987). The effects of CO_2 on *Schiz. pombe* were otherwise the same as the effects of CO_2 on *Sacch. cerevisiae*. The inhibition of cell division by CO_2 has also been observed in algae. After 8 h in 5% CO_2 in air *Chlorella* spp. did not reveal any internal subdivision into daughter cells, despite being shown to be capable of division (Sorokin 1962).

9. Inhibition of other industrial fermentation processes by CO_2

The use of CO_2 for the control of fermentation end-products is not restricted to the brewing industry, and indeed there are many examples in which CO_2 can affect the progress of industrial fermentations (Table 3), for good or for ill. CO_2 pressures may significantly influence the regulation of microbial metabolism favouring biomass or product formation (Mudgett & Bajracharya 1979; Bajracharya & Mudgett 1980).

9.1. EFFECTS OF CO_2 ON THE PRODUCTION OF BIOMASS

The partial pressure of CO_2 may be increased to several atmospheres in some fermenters, but in some cases, for example pressure-cycle fermenters such as the 'Pruteen' plant at Billingham, UK, the partial pressure of CO_2 may fluctuate over a substantial range. In the case of the 'Pruteen' plant the pCO_2 may change from some 0·45 atm to 0·05 atm in 1 min (Vasey & Powell 1984); the CO_2 concentration

Table 3. A summary of some of the industrial fermentation processes that are inhibited by CO_2

Product/process inhibited by CO_2	Selected references
Biomass	
'Pruteen'	Vasey & Powell 1984
Baker's yeast	Chen & Gutmanis 1976
Solvents	Klei *et al.* 1984
Amino acids	
Arginine	Akashi *et al.* 1979; Hirose 1986
Histidine	Akashi *et al.* 1979; Hirose 1986
Glutamic acid	Hirose *et al.* 1968
Inosine	Shibai *et al.* 1973; Ishizaki *et al.* 1973
Antibiotics	
Penicillin	Ho & Smith 1986
Tetracycline	Tikhonov *et al.* 1983
Oleandomycin	Bylinkina *et al.* 1973
Streptomycin	Bylinkina *et al.* 1973
Methane	Hansson 1979
Degradation of lignocellulosic wastes	Drew & Kadam 1979; Mudgett & Paradis 1985

These processes are discussed in detail in the text of section 9.

is at its highest close to the base of the riser, where growth is the greatest. Growth of the organism used for the 'Pruteen' process, *Methylophilus methylotrophus*, was found to be sensitive to CO_2, with the maximum specific growth rate decreasing from 0·5 to 0·15/h as the partial pressure of CO_2 increased from 0·05 to 0·4 atm. Above 0·29 atm CO_2 the product specification changed and the carbon-to-cell conversion decreased (Vasey & Powell 1984).

During biomass production with bakers' yeast, inhibition of the extent of yeast growth below 0·2 atm pCO_2 in the gas phase was negligible, with slight inhibition at 0·4 atm and significant inhibition at 0·5 pCO_2 (Chen & Gutmanis 1976). In the case of ethanol production by *Zymomonas mobilis* biomass production is inhibited by CO_2 (Schreder *et al.* 1934) and it was noted that nucleation, by adding diatomaceous earth, or additional stripping of CO_2, increased the glucose uptake rate during the early stages of fed-batch fermentation (Burrill *et al.* 1983). During continuous cultivation, at high dilution rates, biomass production was increased by as much as 100% when CO_2 was decreased from 1·46 atm to 0·095 atm (Nipkow *et al.* 1985). Product yield was not affected by CO_2 partial pressure, but the glucose uptake and ethanol production rates decreased as the partial pressure of CO_2 was decreased. Nitrogen sparging was found to reduce lag times considerably in batch cultures, probably because of the removal of CO_2 (Veeramallu & Agrawal 1986). In this study, however, no noticeable trend in glucose uptake or ethanol production rates were observed when CO_2 was removed from the culture medium. Despite this the specific growth rate increased by 15% and the cell mass yield increased by 12%, but the overall ethanol yield decreased by 5%. Production of CO_2 was shown to be directly coupled with ethanol formation but not necessarily with cell mass production, indicating a decoupling of growth from ethanol production (Veeramallu & Agrawal 1986). It was noted that high pCO_2 combined with high ethanol concentrations caused a change in the morphology of *Z. mobilis*, leading to the appearance of extensive slime and granular layers around the cells (Doelle & McGregor 1983). Alteration of the cellular morphology has also been obtained with *Strep. mutans*, the shape of which could be dictated by the ratio of 'CO_2', in the form of bicarbonate, to K^+ in the growth medium (Tao *et al.* 1987). A high bicarbonate/K^+ ratio produced spherical cells, whereas the cells remained bacillary in medium with a low bicarbonate/K^+ ratio.

9.2. EFFECTS OF CO_2 ON THE PRODUCTION OF BIOCHEMICALS

During the production of acetone and butanol by *Cl. acetobutylicum*, the butanol/acetone ratio is significantly changed at CO_2 pressures above 2·6 atm, with the virtual elimination of ethanol production (Klei *et al.* 1984). Maximum solvent production by *Cl. acetobutylicum*, at the expense of cell growth, was found to occur at 1·7 atm and substrate utilization was inhibited with increasing pCO_2. The ability to shift the product distribution would allow the fermentation plant manager to respond to changes in demand and price. In contrast to the effects of CO_2 on the product ratios of *Cl. acetobutylicum*, the partial pressure of CO_2 does not noticeably influence the fermentation products of *Cl. butyricum* (van Andel *et al.* 1985).

During aerobic batch growth of *Brevibacterium flavum* on glucose under controlled gas phases, CO_2 was found to affect amino acid production (Akashi *et al.* 1979; Hirose 1986). At all pCO_2 values CO_2 reduced the yield of histidine, produced by *Brev. flavum*, whilst arginine displayed an optimal yield at a pCO_2 of 0·12 atm, above which the yield decreased rapidly. In this instance, as in the case of the fermentation by *Cl. acetobutylicum*, it is thus possible to control the ratio of products by the use of CO_2.

During glutamic acid production by *Brev. lactofermentum* high partial pressures of CO_2 caused a decrease in glutamate production, sugar consumption and respiratory activity (Hirose *et al.* 1968). High CO_2 tension also resulted in a decrease in product formation in a nucleoside fermentation employing a mutant of *B. subtilis* (Shibai *et al.* 1973). In the same fermentation, the yield of inosine was shown to be independent of the bicarbonate ion concentration in the culture medium, but greatly reduced by increasing partial pressures of CO_2 (Ishizaki *et al.* 1973).

The production of antibiotics is inhibited by relatively low partial pressures of CO_2. The rate of synthesis of penicillin by *Pen. chrysogenum* was halved by a CO_2 partial pressure of 0·08 atm (Pirt & Mancini 1975), when compared with the rate of synthesis at 0·006–0·007 atm pCO_2. Exposure to influent gases of 0·03 and 0·05 atm of CO_2 produced no observable inhibition of the metabolism of *Pen. chrysogenum*, but influent gases of 0·126 and 0·2 atm of CO_2 inhibited both growth rate and penicillin production rate (Ho & Smith 1986). Tetracycline biosynthesis was found to be optimal at 0·02 atm CO_2 (Tikhonov *et al.* 1983) and the inhibition by CO_2 of the production of other antibiotics, such as oleandomycin and streptomycin, have also been reported (Bylinkina *et al.* 1973).

9.3. EFFECTS OF CO_2 ON THE TREATMENT OF WASTES

Any inhibitory effect of CO_2 could be of great importance in methane production during the digestion of wastes, since the partial pressure of CO_2 could increase to several atmospheres in large digestors. In general, rapid increases in pCO_2 resulted in rapid decreases in methane production and vice versa (Hansson 1979). The methane yields were 20–30% higher at low values of pCO_2 compared with those at 1 atm CO_2 (Hansson & Molin 1981). Combined with decreasing methane yields, as pCO_2 increases from 0 to 1 atm, there is a progressive inhibition of acetate and propionate degradation, although some CO_2 was required for propionate degradation since the rate of degradation dropped rapidly below 0·2 atm CO_2 (Hansson & Molin 1981; Hansson 1982). High pCO_2 also decreased the temperature maximum at which bacterial acetate degradation occurred (Hansson 1982).

Conversion of lignocellulosic wastes is also susceptible to inhibition by CO_2; increasing CO_2 pressure increasingly inhibits the rate and extent of degradation of lignin and non-lignin materials by *Phanerochaete chrysosporium* (Mudgett & Paradis 1985). Lignin degradation rates were also suppressed by high levels of CO_2 in the later stages of the fermentation catalysed by *Aspergillus fumigatus* (Drew & Kadam 1979). Both inhibitory and stimulatory effects of CO_2 have been reported for a number of other fermentations (Mudgett 1980). It may be concluded therefore that in some cases CO_2 exerts a controlling influence upon microbial growth and metabolism which may be utilised for biotechnological purposes. It is therefore germane to enquire into some of the possible mechanisms whereby CO_2 exerts its inhibitory effects at the subcellular level.

10. Sites of action

10.1. BIOLOGICAL MEMBRANES

As it has been noted above that CO_2 may inhibit cell division and cause alterations in cell morphology, glucose uptake rates and amino acid absorption, it would be reasonable to suggest that such effects of CO_2 may be associated with changes in the function of the biological membrane. Indeed one of the factors implicated in contributing to the reason for the growth-inhibitory effects of CO_2 has been the alteration of membrane properties (Sears & Eisenberg 1961). It was suggested that CO_2 interacts with lipids of the cell membrane, decreasing the ability of the cell to uptake various ions. 'Anaesthesia' (i.e. narcosis) caused by elevated levels of pCO_2 was proposed as a basis for the effect of CO_2 on the membrane during CO_2-mediated growth inhibition, and the available literature on yeast was reviewed (Jones & Greenfield 1982). Changes in the permeability of the cell membrane were also invoked in connection with a study of the effects of CO_2 on the germination of bacterial spores (Enfors & Molin 1978b). The inhibition was suggested to be due to an increase in fluidity, causing the disturbance of the activity of a membrane-bound enzyme essential to the initiation of germination. Alterations in the fatty acid content, and fluidity, of the yeast cell membrane at elevated pCO_2 levels were noted (Castelli *et al.* 1969).

One theory of narcosis is that absorption of an 'anaesthetic agent' into a biological membrane causes a hydrophobic region to expand beyond a certain critical size. This theory is termed the critical volume hypothesis (Miller *et al.* 1973) and is consistent with the observations that anaesthetics expand monolayers, bilayers and bulk solvents (Seeman 1972) and that hydrostatic pressure can in some cases reverse anaesthesia (Lever *et al.* 1971). Expansion of biological membranes at clinically significant concentrations of general anaesthetic is estimated to be of the order of 0·4% (Lever *et al.* 1971). It has been demonstrated that anaesthetics disorder the lipid bilayer of the membrane (Jain *et al.* 1975, Vanderkooi *et al.* 1977, Pang *et al.* 1980). Perturbations in membrane fluidity, caused by the disordering of the lipid bilayer, are postulated to alter the function of membrane proteins, resulting in the changes that may be associated with anaesthesia (Chin *et al.* 1976; Roth 1980). The phase transition hypothesis (Lee 1976) suggests that lipids surrounding functional membrane proteins exist in a gel phase (as a lipid annulus) retaining proteins that form ion channels in an open state, and anaesthetics 'melt' this rigid lipid (Roth 1980). Regions in which lipids in the liquid phase coexist with lipids in the gel phase are termed lateral phase separations (Trudell 1977) and are associated with functional changes in membrane proteins, possibly modulating the protein conformation through hydrophobic or electrostatic interactions. Anaesthetics may reduce these lateral phase separations, thus altering the functional activity of the proteins (Roth 1980).

In each of the above theories the anaesthetic is taken to be interacting with the lipid portion of the membrane, and these theories are known as lipid theories of narcosis. In contrast with these theories are the protein theories of narcosis. These latter theories proposed that narcosis is due to a direct effect of the anaesthetic upon membrane proteins (Richards *et al.* 1978; Franks & Lieb 1986). The degenerate perturbation hypothesis (Richards *et al.* 1978) proposes that small molecules are distributed in one set of hydrophobic sites of appropriate dimensions within membrane proteins, and larger molecules may bind to distinct sets of sites on the membrane proteins. In addition to this, molecules with structural features similar to those of phospholipids will compete for the regions of the proteins that interact with phospholipids; the replacement of annular lipids by anaesthetic molecules then interferes with the function of the protein.

n-Alcohols behave as anaesthetics; their potency increases until a certain chain length is reached, beyond which their biological activity ceases. The lipid theories state that this 'cut-off' in anaesthetic activity is due to a corresponding cut-off in the absorption of the alcohols (that are then longer than the specific chain length) into the lipid bilayer of the membrane. However, it has been demonstrated that the partition of alcohols into lipid bilayers continues long after their biological activity has ceased (Franks & Lieb 1986). This supports the view that it is the membrane proteins and not the lipid bilayers, that are the target sites for anaesthetics. The general anaesthetic properties of the *n*-alcohols can be accounted for in terms of binding to protein target sites of circumscribed dimen-

sions: the levelling off in potency occurs when the site becomes full, and the cut-off point occurs when the concentration for anaesthetic activity falls below the aqueous solubility (Franks & Lieb 1986).

Narcosis may also be caused by the action of CO_2 on the cytoplasm as well as on the plasma membrane. During CO_2 narcosis of the filamentous alga, *Nitella clavata*, there is a gradual reversible sol-to-gel conversion of the cytoplasm, assumed to be associated with the carbamination of proteins (Fox 1981). Removal of CO_2 from the gas phase restores the sol state of the cytoplasm from its partially gelled condition. Also involved in the narcosis of the cytoplasm is the exosmosis of water and electrolytes. The carbamination and subsequent coalescence of enzymic proteins would presumably deprive them of their full function as catalysts (Fox 1981).

Reaction of amino groups, present on membrane proteins, with CO_2 will result in potentially positive ions (RNH_3^+) becoming potentially negative ions ($RNHCOO^-$) (Mitz 1979). This charge change causes a change in the surface potential and may selectively favour the transport of positive ions, whilst inhibiting the transport of negative ions across the membrane. In addition to this there would probably be a significant conformational change associated with the charge change, which would also affect the function of the protein.

The inhibition by CO_2 of the uptake of amino acids by membrane vesicles of *E. coli* and *B. subtilis* was only one half that of the inhibition of growth, whereas the uptake of glucose by membrane vesicles of *E. coli* was unaffected by 100% CO_2 (Eklund 1984). This might be interpreted to mean that the main mechanism of CO_2 action is not associated with the biological membrane. Such an interpretation can now be seen to be incorrect when viewed in terms of the so-called metabolic control analysis (e.g. Kacser & Burns 1973; Westerhoff *et al.* 1984), which is discussed in the next section.

10.1.1. *The metabolic control analysis*

The metabolic control analysis was originally formulated to describe the control of metabolic pathways. When considering the control of metabolic pathways it is traditional to ask the question 'which step is rate limiting?', when referring to pathway substrates or products. 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 formalism with which quantitatively to approach such a question is the metabolic control theory (see e.g. Kacser & Burns 1973; Kell & Westerhoff 1986a, b, Kacser & Porteus 1987; Westerhoff & Kell 1987, Kell *et al.* 1989). 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

$$C_i^J = [(\mathrm{d}J/\mathrm{d}e_i) \times (e_i/J)]_{ss} \qquad (11)$$

$$= (\mathrm{d} \log |J|/\mathrm{d} \log e_i)_{ss}$$

where C_i^J is the flux 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 the 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. The control analysis contains a number of other theorems relating system properties such as fluxes to the properties (the so-called elasticities) of individual enzymes. The metabolic control theory can be applied to processes other than flux through metabolic pathways, as in our work on the effects of CO_2 upon the growth rate of *Cl. sporogenes* (Dixon *et al.* 1987). Hence the above statement, that the main mechanism of CO_2 action is not associated with the biological membrane, may be incorrect, as this mechanism will contribute to the overall effect of CO_2 on the cell. If the control coefficient on growth for a membranous enzyme (times the elasticity of the same enzyme towards CO_2) is greater than that for any other similar interaction, then it will in fact be the 'main' mechanism of action. Further, the value of the

control coefficient will tend to vary with the environmental conditions, so in one set of conditions one enzyme may exert the most control, but in another set of conditions it may be another enzyme that exerts the most control on the growth rate.

10.2. CYTOPLASMIC ENZYMES AS A SITE OF ACTION OF CO_2

Rather than a generalized effect on membrane-located proteins, CO_2 may exert its influence upon a cell by affecting the rate at which *particular* reactions proceed. One way in which this may be brought about is of course to cause an alteration in the production of a specific enzyme or enzymes, via induction or repression of enzyme synthesis (Jones & Greenfield 1982; Sarles & Tabita 1983; Bowien & Leadbeater 1984; Dixon 1988). It was suggested (Wimpenny 1969), and evidence has since been obtained (Wood & Stjernholm 1962; Wood & Utter 1965; Wimpenny 1969; Jones & Greenfield 1982), that the primary sites at which CO_2 exerts its effects are the enzymatic carboxylation and decarboxylation reactions, although the effects extend to enzymes not necessarily involved in carboxylation or decarboxylation reactions (Jones & Greenfield 1982).

There have been a number of studies showing that CO_2 causes alterations in the rate of certain enzymatic reactions in bacteria, leading to the build-up of the concentrations of certain metabolites. Thus, increasing the concentration of CO_2 caused the rate of succinate formation by *E. coli* var. *commune* to increase, the removal of CO_2 causing a decrease in the succinate concentration (Elsden 1938). pCO_2 values of 0·01 to 0·1 atm affected the enzymes of *Sclerotium rolfsii*, causing a decrease in the succinate dehydrogenase activity and significant increases in the isocitrate lyase, isocitrate dehydrogenase, malate synthase and malate dehydrogenase activities, compared to when the organism was grown in air (Kritzman *et al.* 1977).

10.2.1. *CO_2-controlled induction/repression of enzyme synthesis*

The control exerted upon the enzymes of *Sclerotium rolfsii*, as mentioned above, was through induction/repression of enzyme synthesis. Similarly the control of autotrophic CO_2 fixation by CO_2 is mostly by induction or repression of enzyme synthesis. When CO_2 was supplied at low partial pressures (=0·02 atm in hydrogen) to photolithotrophically grown cells of *Rhodospirillum rubrum*, up to 50% of the soluble protein was ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCase); increasing the pCO_2 from 0·02 to 0·05 atm resulted in a rapid and dramatic decrease in the rate and extent of RuBP synthesis. Lowering the CO_2 back to 0·02 atm resulted in a dramatic increase in RuBPCase synthesis (Sarles & Tabita 1983; Tabita *et al.* 1985). This response to the partial pressure of CO_2 appeared to be typical of other chemotrophic and phototrophic organisms, although they may not be as sensitive to CO_2 or synthesize the same amount of RuBPCase as *Rhodo. rubrum* (Sarles & Tabita 1983; Tabita *et al.* 1983, 1984). CO_2 limitation of *Alcaligenes eutrophus* depresses the synthesis of RuBPCase and phosphoribulokinase (PRK) to about one-fifth of the normal autotrophic level (Friedrich 1982; Bowien & Leadbeater 1984). RuBPCase is activated by CO_2 (Lorimer 1981a; Miziorko & Lorimer 1983). The reversible activation process is accompanied by a carbamate formation at a specific activator site lysine on the large subunit of the enzyme (Lorimer & Miziorko 1980; Lorimer 1981b). Activation of RuBPCase from *Alc. eutrophus* was shown to result in a significant change of the hydrodynamic properties of the enzyme. The inactive enzyme exhibited a sedimentation coefficient, $S_{20,w}$, of 17·5 S, whereas activation lowered this value to 14·3 S (Bowien & Gottschalk 1982). This alteration reflects major changes in the tertiary structure of the enzyme, in which the small subunits could have a crucial function. For further information about the control of CO_2 fixation see also Tabita (1981), Dijkhuizen & Harder (1984) and Bowien *et al.* (1987).

10.2.2. *CO_2 inhibition of enzyme reactions*

Another way in which CO_2 may influence the rate of a reaction is to inhibit or stimulate the reaction, rather than affecting the synthesis of the appropriate enzyme. Formate hydrogenlyase of *E. coli* was also inhibited by CO_2 (Swanson & Ogg 1969). Fumarate formation by *Rhizopus nigricans* was

blocked at the pyruvate carboxylation step due to the inhibition of oxaloacetate decarboxylase activity by CO_2 (Foster & Davis 1949). Both malate dehydrogenase and isocitrate dehydrogenase of *Ps. aeruginosa* were found to be inhibited by CO_2, but oxaloacetate decarboxylase, fumarase, succinate dehydrogenase and cytochrome *c* oxidase were unaffected, although a small amount of inhibition might have been undetected (King & Nagel 1975). Catalase activity was reversibly decreased by half in 0·13 atm pCO_2 (Bretskin & Ivanova 1955), although the peroxidatic activity of the enzyme was enhanced (Mitsuda *et al.* 1958). The extracted protease activity of *Ps. fraqi* was inhibited by increasing concentrations of CO_2, whereas in the case of *Streptomyces caespitosus* the extracted protease activity exhibited an optimum pCO_2 of 0·4 atm pCO_2 in argon as compared with its activity in pure argon (Pichard *et al.* 1984).

11. Mechanisms of CO_2 inhibition of microbial growth and metabolism

Despite numerous reports of the effects of CO_2 on microbial growth and metabolism, a number of which have been discussed, the 'mechanism' of CO_2 inhibition still remains unclear. From what has been written above, a unitary mechanism of CO_2 inhibition seems out of the question. However, a number of possible explanations have been postulated.

In an anaerobic chemostat culture of *Klebsiella aerogenes* it was demonstrated that increased concentrations of metabolically produced CO_2 effected a lowering of yield values (Teixeira de Mattos *et al.* 1984). CO_2 exerts an effect on both metabolism and the energetics of cell synthesis. In this case the proposed mechanism was one of a futile cycle, dissipating energy, stimulated by increased CO_2 concentrations and involving carboxylation and decarboxylation reactions, the net result being $ATP \rightarrow ADP + P_i$ (Teixeira de Mattos *et al.* 1984 and see Fig. 1a). Such effects of CO_2 on growth energetics have been observed with other organisms. During the growth of *Cl. sporogenes* in 'glucose-limited' chemostat culture in a defined minimal medium in which this organism required CO_2 for growth (Dixon *et al.* 1987; Lovitt *et al.* 1987), CO_2 induced some type of metabolic 'slip', since both the yield coefficient *and* the apparent maintenance requirements decreased as the pCO_2 was increased above the optimal pCO_2 (0·5 atm) for growth rate in 'unrestricted' batch culture (Dixon *et al.* 1988; see also Pennock & Tempest 1988 for similar energetic behaviour in *B. stearothermophilus*). A futile cycle stimulated by CO_2 and pH, involving the CO_2/HCO_3^- equilibrium was proposed as a possible mechanism of energy dissipation in the former case (Fig. 1b). The occurrence of futile cycles will have an increasing importance, to the cell, as the growth rate is decreased e.g. in nature.

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). When *Cl. sporogenes* was grown in chemostat culture in a medium in which CO_2 was not required for growth (Dixon *et al.* 1987; Lovitt *et al.* 1987), as the pCO_2 was increased above the optimal pCO_2 for growth rate in 'unrestricted' batch culture, the thermodynamic efficiency of growth was increased (in that both yield (Y_{ATP}^{max}) and maintenance requirements increased (*sic*)); thus the system was no longer optimal for maximum growth rate (Dixon *et al.* 1988).

Variation in the response to CO_2 with medium composition was observed in the case of *Ps. fluorescens* (Tan & Gill 1982). If basic cell functions, such as protein synthesis, or reactions of central metabolic pathways were the processes that became most rate-limiting for growth under CO_2, then the pattern of inhibition would be expected to be similar in the different media. As they were not it was necessary to postulate different rate-limiting reactions for each medium. It was postulated that the inhibition of growth rate by CO_2 would depend on either non-specific inhibition of enzymes involved in the early stages of substrate utilization or the inhibition of substrate uptake. Indirect inhibition of enzymes, resulting from a decrease in intracellular pH caused by CO_2 (Wolfe 1980), was untenable as an explanation since decreasing the intracellular pH by altering the medium pH actually stimulated growth (Tan & Gill 1982). The conclusion arrived at was that the inhibitory action of CO_2 was probably exerted at the level of substrate uptake. A point in favour of this conclusion was the failure of other workers to detect an accumulation of metabolites during CO_2-inhibited growth of *Ps.*

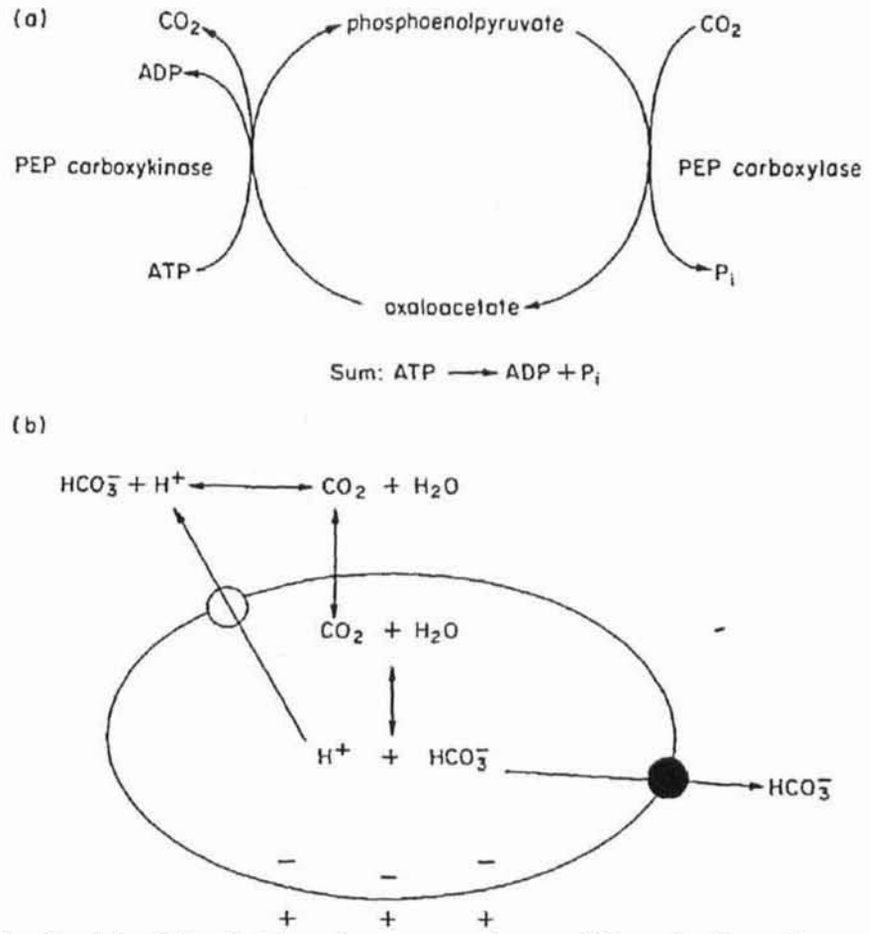

Fig. 1. Futile cycles, involving CO_2, that have been proposed as possible mechanisms of energy dissipation. (a) The futile cycle proposed for *Klebsiella aerogenes* (Teixeira de Mattos *et al.* 1984) which involves PEP carboxylase and PEP carboxykinase and is stimulated by CO_2. (b) CO_2 freely permeates the biological membrane and 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.

aeruginosa (King & Nagel 1975), an organism closely related to *Ps. fluorescens*. Accumulation of some intermediate might be expected to result from the inhibition of specific catabolic enzymes, but this would not occur if the growth rate was being determined by the rate of substrate uptake. However, metabolites might not accumulate due to increased metabolic flux through other pathways that do not include CO_2 inhibited reactions. Additionally, substrate uptake may be expected to contribute to the growth rate-limitation but not be the sole reason for such limitation (see Dixon *et al.* 1987; Walter *et al.* 1987).

During the storage of proteinaceous foods in modified atmospheres containing CO_2, the interaction of CO_2 is achieved through solubilization and absorption of CO_2 (Mitsuda *et al.* 1975). Most probably, a major factor in the efficacy of CO_2 lies in its ability to penetrate the bacterial membrane, causing intracellular pH changes (Aickin & Thomas 1975; Turin & Warner 1977) of a greater magnitude than would be found for similar external acidification, which can be effectively buffered by the organism. The pH changes induced by medium-to-high partial pressures of CO_2 in the storage atmospheres are sufficient to disrupt the internal enzymatic equilibria (Wolfe 1980). Such internal pH changes may affect enzymes not otherwise involved with CO_2, and in addition to this, as all decarboxylation reactions appear to produce carbon dioxide in the $CO_{2(aq)}$ form (Krebs & Roughton

1948; Delente *et al.* 1969) there is the possibility of feedback inhibition at elevated pCO_2. Reducing the temperature would serve to enhance this function by increasing the solubility of CO_2.

Mutants of *E. coli*, *Neurospora crassa* and *Salm. typhimurium* that are specifically inhibited by CO_2 have been isolated (Roberts & Charles 1970). These mutants are inhibited at CO_2 concentrations which stimulate CO_2-requiring mutants and which do not inhibit the wild-type organisms, with the inhibition being relieved by the addition of specific growth substances; for example, the CO_2 inhibition of a methionine-requiring mutant of *N. crassa* is reversed by purines, and the CO_2 inhibition of a prototroph of *E. coli* is reversed by methionine or vitamin B_{12}.

Carbon dioxide may exert a direct influence upon enzymes by affecting their physico-chemical properties. The rate and extent of solution of proteins in water may be increased by CO_2, and in some cases insoluble proteins become soluble; this is reversible since they precipitate out again upon removal of the CO_2 (Mitz 1957, 1978). Hence CO_2 may be utilized to adjust the solubility of proteins during their separation and purification (Yanari *et al.* 1960). Changes in CO_2 tension may cause changes in the rate of solution, absolute solubility, dissociation of complexes, reactivity, stability, charge and configuration of proteins (Mitz 1978).

A property of CO_2, mentioned above, is that it has a high reactivity with amines (Edsall 1969). The reaction of CO_2 with uncharged primary amines is many times faster than with water, resulting in the formation of a carbamic acid (RNHCOOH), with different amines having different reaction rate constants. At equilibrium the amount of carbamate is small, as the dominating reaction is the reaction with water, due to the relatively small concentrations of the proteins and the high concentration of water present.

As has been indicated CO_2 can react with amino acids, peptides and proteins of the cell. The protein carbamate can cause the formation of internal electrostatic attractions or repulsions that could result in structural changes (Mitz 1979). These interactions could be between separate portions of the protein, between sub-units of a protein complex, or between the protein and bound heavy metals (Mitz 1979). Charge changes due to the reaction of CO_2 with amino groups, as mentioned above in the case of membrane proteins, could cause very important changes in the properties of the protein, especially in the case of enzymes which depend upon the possession of a specific charge for their activity. It is these structural and charge changes that may be responsible for the above-mentioned changes in the physico-chemical properties of proteins, caused by CO_2.

Alteration of the physico-chemical properties of a protein has been demonstrated in the case of haemoglobin (Kilmartin & Rossi-Bernardi 1973). CO_2 attached to the amino group of the protein as a carbamate is associated with structural changes of the molecule which decrease the affinity for O_2. The ability of CO_2 to modulate the activity of enzymes has been demonstrated on enzymes *in situ*, in the case of enzymes of carbohydrate and fatty acid metabolism of rat liver (Hastings 1970; Longmore *et al.* 1974), as has the ability of CO_2 or bicarbonate to regulate enzyme activity in crude cell-fractions, e.g. $NADP^+$-specific glycerol dehydrogenase (Legisa & Mattey 1986). Another example of this is the activation of RuBPCase by CO_2, mentioned above, resulting in the alteration of the enzyme's sedimentation coefficient (Bowien & Gottschalk 1982).

12. Concluding remarks

Despite the large number of reports upon the inhibition by CO_2, which forms the basis of the discussion above, there does not appear to be a clear understanding of the role of CO_2 in the growth and metabolism of any given organism. Systematic studies of the effects of CO_2 on bacteria are therefore warranted. Such studies are of importance in attempting to understand: (1) the role of CO_2 in the preservation of foodstuffs, (2) the control by CO_2 of fermentations of biotechnological importance, and (3) the production of fine chemicals *via* reactions involving CO_2.

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