7
The Bacterial Utilization of Synthetic Gases Containing Carbon Monoxide

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The occurrence and industrial uses of synthetic gases containing carbon monoxide

Synthetic gases containing carbon monoxide (CO) and hydrogen (H_2) are extremely important industrial feedstocks for the chemicals industries (*Table 1*). They may be manufactured from coal, lignite or peat which currently account for about 47% of the total energy value of the world's known recoverable reserves of fossil fuels (Gibbs and Greenhalgh, 1983). Gasification of coal for the production of chemicals has been used for more than 40 years and is particularly important in South Africa where the Sasol Company meet internal gasoline and diesel oil demands using the Fischer–Tropsch process. Coal is initially broken down to simple units by gasification and these are reacted to form more complex products. The most important gasification processes produce synthesis gas, predominantly a mixture of CO and H_2, the proportions varying with the gasification process and the type and quality of the coal used. Gasification is achieved by reacting coal with steam and air or oxygen at temperatures greater than 750°C at normal or elevated pressures. A variety of processes are used, usually employing either fixed bed or fluidized bed techniques. Synthesis gas may be converted to a mixture of H_2 and carbon dioxide (CO_2) via the water gas shift reaction, methane (substitute natural gas), methanol (for use as a chemical feedstock or as a carbon and energy source for single-cell protein production by the ICI Pruteen process), ammonia by the Haber–Bosch process and hydrocarbons by the Fischer–Tropsch process.

Although improvements in efficiency of gasification and reduction of costs have recently been achieved by the British Gas–Lurgi Slagging Gasifier and

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Table 1. Composition of industrial gases containing carbon monoxide

	Composition (%)					
Gas	CO	CO_2	H_2	N_2	CH_4	Trace gases
Water gas	22–42	8–18	8–39	1–56	1	H_2S
Synthesis gas	50	None	50	None	None	H_2S
Lurgi gas	28–30	1	56–60	2	7–13	O_2

References: Schmidt (1950); Hollemann and Wiberg (1971); Meyer (1980).

by the use of heat and steam generation from nuclear power, the widespread availability and use of synthesis gas may depend on improvements in underground gasification of coal deposits. Underground gasification may lead to abundant and secure supplies of synthesis gas by exploiting offshore reserves and seams found at extreme depths of more than 2000 metres (Medaets, 1980). This process has been investigated in the USSR since 1934, and three sites have been continuously producing synthesis gas from coal and lignite deposits at depths of between 100 and 300 metres for over 17 years (Gibbs and Greenhalgh, 1983). The technique of *in situ* gasification consists of connecting two or more boreholes via an underground circuit and forcing air or oxygen through under high pressure. Temperatures between 800°C and 1000°C are generated in the coal seam and the volatile products are collected from the boreholes. Scientists in the USA are developing this technique for gasification of deposits of other types of fossil fuels such as oil, oil shales and tar sands. Although trials in Belgium and the UK were abandoned in the 1950s because of poor results and technical difficulties, a new three-stage process using high and cyclical changes in pressure to improve the efficiency of extraction of gases is being developed in Belgium and West Germany to operate at depths of 1000 metres (Becker, 1982). One problem is that the deeper the seam lies, the further apart the two boreholes must be (for economic reasons) and the costs increase as it becomes more difficult to complete the circuit efficiently. At 1000 metres the boreholes must be 80 to 100 metres apart. In experiments at Thulin, Belgium, the efficiency of extraction was increased by passing compressed air through the seam to increase the size of the natural fissures (Becker, 1982).

The properties and natural cycle of carbon monoxide

Carbon monoxide is a colourless, odourless, tasteless, explosive and extremely toxic gas. It has an ignition temperature in air of 700°C within the flammable limits of 12–75%. Solubility in water is low (3·3 ml/100 ml water at 0°C and 2·3 ml/100 ml water at 20°C) and this limits its availability as a substrate for fermentation processes, particularly at high temperatures. Carbon monoxide is a stable triply-bonded molecule with one sigma and two pi bonds (Hughes, 1985). It is extremely toxic because it forms complexes with

transition metals using the unfilled d orbitals of the metal and in man it readily binds to haemoglobin to form carboxyhaemoglobin, preventing oxygen binding and transport. The safety limit for exposure to CO set by the US Environmental Protection Agency is 9 ppm for 8 hours of exposure.

Atmospheric concentrations of CO are about 0·1 ppm in clean rural districts, but can be as high as 100–200 ppm in polluted urban districts. The annual input of CO into the atmosphere from natural and anthropogenic sources has been estimated as $1{\cdot}4 \times 10^9$ tonnes, but atmospheric concentrations remain fairly constant, suggesting that removal occurs at approximately the same rate. The removal of CO from the atmosphere is rapid and a mean residence time of 0·1 to 1·0 year has been estimated (Seiler and Junge, 1970). Anthropogenic sources of CO are varied, but mainly result from incomplete combustion processes. Blast furnace gas (25–30% CO), automobile exhausts (0·5–12% CO) and faulty domestic heating are the main sources, producing approximately 6×10^8 tonnes/year (Meyer, 1980). Man also produces small amounts from the oxidation of haemoglobin to equimolar amounts of CO and biliverdin by microsomal haem oxygenase (Landaw, 1970). This is released through the lungs and may accumulate in enclosed environments, such as in submarines. Man is also exposed to the poisonous effects of CO by smoking cigarettes (2–5% CO), cigars and pipe tobacco (5–14% CO), habits which may lead to the development of circulatory problems, contributing to heart disease. The other main abiogenic sources are direct release from the earth's crust, volcanic gases (1–4% CO), the photochemical oxidation of methane by the hydroxyl ($^{\cdot}OH$) radical and oxygen (4×10^8 tonnes/year), and bush fires (Seiler, 1974, 1978).

Biogenic sources include light-dependent production in some plants (1×10^8 tonnes/year), green algae, brown algae (up to 10% CO in the gas vacuoles), cyanobacteria and anaerobic phototrophic bacteria (Langdon, 1917; Loewus and Delwiche, 1963; Troxler, 1972; Troxler and Dokos, 1973; Bauer, Conrad and Seiler, 1980). The mechanism of production by phototrophic organisms is not clear. In cyanobacteria it has been shown to be a by-product of the biosynthesis of phycobilins from haem precursors (Troxler, 1972; Troxler and Dokos, 1973). Other sources are the decomposition of porphyrins, the catabolism of haem, cobalamin and flavanoids by bacteria and fungi, the peroxidative degradation of lipids, and the production by yeasts, bacteria and fungi during growth (Westlake, Roxburgh and Talbot, 1961; Engel *et al.*, 1972, 1973; Radler *et al.*, 1974). Carbon monoxide is also released from submarine hydrothermal systems, probably resulting from the activity of thermophilic bacteria and is produced by some marine invertebrates (Baross, Lilley and Gordon, 1982).

The main mechanisms for the removal of CO from the upper atmosphere are photochemical destruction in the stratosphere and by oxidation with hydroxyl radicals, according to the equation:

$$CO + {}^{\cdot}OH = CO_2 + {}^{\cdot}H$$

It has been estimated that photochemical processes remove up to 10% of the total (Seiler and Junge, 1970), and oxidation by hydroxy radicals between 50% and 80% (Weinstock and Niki, 1972). The main reservoirs for the remaining CO are the oceans, fresh waters and soils. Although the oceans function as an important reservoir for CO, it has been estimated that they are responsible for a net production of 10% of the total global output (Swinnerton, Linnenbom and Lamontagne, 1970). In the euphotic layer, where CO is >30-fold that expected from atmospheric concentrations, there is a marked diurnal effect which is dependent on the radiation intensity and the concentration of organic matter (Seiler and Junge, 1970). Production is mainly by photo-oxidation of organic matter and is not a result of aerobic microbial metabolism, as the process is insensitive to azide (Conrad and Seiler, 1980). However, below the thermocline there is a low but constant rate of production of CO, probably the result of microbial activity (Swinnerton, Linnenbom and Lamontagne, 1970). A similar pattern emerges from studies of oligotrophic fresh-water habitats. In eutrophic fresh waters microbial metabolism was shown to be the major mechanism for the production of CO. In the anaerobic hypolimnion, the concentration of CO was up to 30 times the concentration found in the oceans, and was associated with high numbers of micro-organisms (Swinnerton, Linnenbom and Lamontagne, 1970). Soil is also an important reservoir for CO. Conrad and Seiler (1980) have suggested that the production of CO in soils is mainly a result of chemical processes and although the rate of production is stimulated by high numbers of micro-organisms, this results from lysis of cells and the subsequent oxidation of the liberated material.

Anaerobic microbial metabolism may have a significant role in the production of CO in soils and fresh waters. Yagi and Tamiya (1962) demonstrated the formation of CO in anaerobic conditions using cell extracts of *Desulfovibrio desulfuricans*. Carbon monoxide has also been shown to be produced during growth of the methanogenic bacterium, *Methanobacterium thermoautotrophicum* (Conrad and Thauer, 1983). A pathway was proposed in which two molecules of CO_2 are fixed into acetyl-CoA by the activity of CO dehydrogenase. The CO_2 is reduced to a bound form of CO which can exchange with free CO (Stupperich *et al.*, 1983). The wide distribution of CO dehydrogenases suggests that this process may occur in many groups of anaerobic micro-organisms.

Carbon monoxide generated in the lower atmosphere and in the oceans, fresh water and soils is mainly consumed locally by micro-organisms (Conrad and Seiler, 1980). Estimates of the global efficiency of removal of CO by soil micro-organisms vary from 1×10^8 t (Heichel, 1973), $4{\cdot}1 \times 10^8$ t (Bartholomew and Alexander, 1979), 5×10^8 t (Liebl and Seiler, 1976; Seiler, 1978), $1{\cdot}4 \times 10^9$ t (Ingersoll, Inman and Fisher, 1974), to $1{\cdot}1 \times 10^{10}$ t (Inman, Ingersoll and Levy, 1971). Conrad and Seiler (1980) demonstrated that the addition of antibiotics to soils severely inhibited CO consumption, indicating the importance of micro-organisms in this process. The relative contributions of bacteria and fungi in various soil types were determined by adding antibiotics specific for either prokaryotic or eukaryotic organisms.

Contrasting views of the metabolic processes involved in the consumption of CO by soil microbial populations have been expressed. Bartholomew and Alexander (1979) observed that only small quantities of added ^{14}CO appeared in the organic fraction of soils and that $^{14}CO_2$ fixation was not stimulated by the addition of CO, suggesting that co-oxidation by non-specific mechanisms was responsible. However, Spratt and Hubbard (1981) demonstrated a higher rate of consumption of CO by roadside soils compared with soils from outlying areas and a marked enhancement of CO_2 fixation in the presence of CO. Microbial populations with a high affinity for CO and capable of utilizing trace amounts of the gas (0·5–1·0 ppm) have been demonstrated, confirming the view that micro-organisms in soils are capable of utilizing CO chemoautotrophically (Conrad and Seiler, 1980).

Bacterial utilization of carbon monoxide

A large number of individual bacterial species belonging to several genera have been shown to utilize CO (Hegeman, 1980; Kim and Hegeman, 1983), either as an energy source (*Table 2*), or by co-oxidation using non-specific enzyme mechanisms which provide no cell energy. Some biologically important reactions involving CO and H_2 are summarized in *Table 3*.

AEROBIC BACTERIA

Co-oxidation of CO by methanotrophic bacteria (Hubley, Mitton and Wilkinson, 1974; Ferenci, 1976), *Actinoplanes, Agromyces, Microbispora* and *Mycobacterium* (Bartholomew and Alexander, 1979) may be common in soils and waters. The process is defined by Anthony (1982) as 'the transformation of a compound, which is unable to support cell replication, in the requisite presence of another transformable compound (co-substrate)'. Co-oxidation of CO to CO_2 in the methanotrophs provides no cell energy and occurs only in the presence of formaldehyde as a source of reductant (NADH). It is a property of the extraordinary lack of enzyme specificity shown by methane monooxygenase (EC 1.14.13.25; Dalton, 1980; Anthony, 1982). The ammonia-oxidizing bacteria *Nitrosomonas europaea* and *Nitrosococcus oceanus* also co-oxidize CO to CO_2 by a non-specific ammonia monooxygenase in the presence of ammonia (Jones and Marita, 1983).

GRAM-NEGATIVE MESOPHILIC CARBOXYDOTROPHIC BACTERIA

The aerobic bacteria utilizing CO as an energy source are collectively known as the carboxydotrophic bacteria (*Table 2*). Beijerinck and van Delden (1903) reported an isolate, *Bacillus oligocarbophilus*, which grew better in contaminated laboratory air than in 'pure air'. This description of a CO-oxidizing bacterium was followed by further unsubstantiated descriptions (Kaserer, 1906; Lantzsch, 1922). Haseman (1927) provided the first evidence of bacterial oxidation of CO. He isolated an organism, *Actinomyces oligocarbophilus*, which grew in a predominantly filamentous form on solid

Table 2. Main groups of carbon monoxide-utilizing bacteria

Class	Main examples	Growth on CO	Enzyme used	Aerobe/anaerobe
Carboxydotrophs	*Pseudomonas carboxydovorans* *Pseudomonas carboxydohydrogena* *Pseudomonas carboxydoflava* *Pseudomonas compransoris* *Pseudomonas gazotropha* *Alcaligenes carboxydus* *Arthrobacter* 11/× *Pseudomonas thermocarboxydovorans* *Bacillus schlegelii* *Bacillus* OMT2 *Streptomyces* G26	Yes	CO oxidase	Aerobic
Methanogens	*Methanobacterium thermoautotrophicum* *Methanobacterium barkeri* *Methanobacterium arboriphilicus* *Methanothrix soehngii*	Yes	CO dehydrogenase	Anaerobic
Homoacetogens	*Clostridium pasteurianum* *Clostridium thermoaceticum* *Clostridium thermoautotrophicum* *Butyribacterium methylotrophicum* *Eubacterium limosum* *Acetobacterium woodii*	Yes	CO dehydrogenase	Anaerobic
Sulphate reducers	*Desulfovibrio desulfuricans*	No	CO-activating enzyme hydrogenase	Anaerobic
Photosynthetic bacteria	*Rhodopseudomonas gelatinosa* *Rhodopseudomonas rubrum*	Yes	CO dehydrogenase	Anaerobic

Table 3. Free energy changes of some biologically important reactions involving carbon monoxide, hydrogen or methane

Reaction	Free energy (KJ)
$2CO + O_2 = 2CO_2$	− 514·21
$2CO + O_2 + 2H_2O = 2H_2CO_3$	− 497·42
$2H_2 + O_2 = 2H_2O$	− 474·35
$CO + H_2O = HCOOH$	+ 2·08
$CO + H_2O = CO_2 + H_2$	− 19·93
$CO + NO_3^- = CO_2 + NO_2^-$	− 183·0
$CO + 2NO = CO_2 + N_2O$	− 326·06
$4CO + SO_4^{--} = S^{--} + 4CO_2$	− 198·03
$CO + 2Fe_3^+ + H_2O = CO_2 + 2Fe_2^+ + 2H^+$	− 168·46
$4HCOOH = CH_4 + 3CO_2 + 2H_2O$	− 130·4
$HCO_3^- + H^+ + 4H_2 = CH_4 + 3H_2O$	− 135·6
$4CO + 2H_2O = CH_4 + 3CO_2$	− 211·29
$4CO + 4H_2O = CH_3COO^- + 2HCO_3^- + 3H^+$	− 165·27
$4H_2 + 2CO_2 = CH_3COO^- + H^+ + 2H_2O$	− 115·48

Gases in gaseous state; solid or liquid compounds in aqueous state

minimal medium in an atmosphere containing CO or CO_2/H_2. Carbon monoxide oxidation was quantified by growing cultures in a bell jar and by demonstrating CO_2 production. Kistner (1953) concluded that these results were unsatisfactory as no account was taken of the micro-organisms present in the water in the bell jar and insufficient oxygen was supplied to allow complete oxidation of CO. Kistner (1953) suppressed the growth of contaminating organisms by enriching in an atmosphere containing 70% CO and isolated a Gram-negative rod from sewage sludge which grew on CO as sole carbon and energy source according to the equation:

$$2CO + O_2 \rightarrow 2CO_2$$

He named the organism *Hydrogenomonas carboxydovorans* because of its ability to use H_2 as an energy source, but it was renamed *Pseudomonas carboxydovorans* by Zavarzin and Nozhevnikova (1977).

Interest in converting hydrogen into single-cell protein using cheap gas mixtures containing CO stimulated the isolation of several new species of carboxydotrophs from sewage sludge, muds and polluted streams. Sanjieva and Zavarzin (1971) described a small motile Gram-negative screw-shaped rod which reproduced by budding and formed stellate aggregates held together by slime. The organism was named *Seliberia carboxydohydrogena* but was later renamed *Pseudomonas carboxydohydrogena* after studies by

Meyer, Lalucat and Schlegel (1980) revealed no evidence of budding and few screw-shaped cells. Autotrophic growth on CO required molybdenum, but no organic growth factors were required. Other one-carbon compounds were not used. Sole carbon and energy sources for heterotrophic growth were limited to fumarate, pyruvate, succinate, lactate, malate, acetate, citrate, aspartate, fructose and sucrose (Sanjieva and Zavarzin, 1971; Nozhevnikova and Zavarzin, 1974; Zavarzin and Nozhevnikova, 1977; Meyer, Lalucat and Schlegel, 1980). The DNA G + C content was determined as 58·2 mol% (Zavarzin and Nozhevnikova, 1977).

The first comprehensive investigation of carboxydobacteria was provided by Nozhevnikova and Zavarzin (1973). They isolated several new Gram-negative species from soils and found that growth was improved by the addition of vitamins. In later studies (Nozhevnikova and Zavarzin, 1974; Zavarzin and Nozhevnikova, 1977) four new species were described, namely *Pseudomonas carboxydoflava, Pseudomonas gazotropha, Achromobacter carboxydus* and *Comamonas compransoris*. The taxonomic position of these strains was reinvestigated by Söder (1980) who renamed *Achromobacter carboxydus* as *Alcaligenes carboxydus* and *Comamonas compransoris* as *Pseudomonas compransoris*. Meyer and Schlegel (1983) provided a comprehensive description of these strains; some of the characteristics are summarized in *Tables 4* and *5*. Meyer and Schlegel (1978) isolated four strains OM2, OM3, OM4 and OM5 from samples of soil and waste water. The properties of OM5 were similar to those described by Kistner (1953, 1954) for *Hydrogenomonas (Pseudomonas) carboxydovorans*. OM5 was therefore named *Pseudomonas carboxydovorans*. The main characteristics of this strain are presented in *Tables 4* and *5*.

There are several reports of carboxydobacteria capable of fixing atmospheric dinitrogen. Ooyama and Shinohara (1971) and Shinohara and Ooyama (1972) isolated two strains from oily soil. The strain designated S17 grew well with CO/H_2 or CO_2/H_2 but not with CO alone, indicating that the CO was used as a carbon source but not as an energy source. The second strain, A305, grew with CO as sole carbon and energy source. Both strains used dinitrogen as nitrogen source during autotrophic growth, but low partial pressures of CO and oxygen were required (Ooyama, 1976). Four strains of Gram-negative bacteria capable of using CO as sole carbon and energy source and of fixing dinitrogen were isolated from soil (Kirkconnell and Hegeman, 1976; Kirkconnell, 1978). One strain was assigned to the genus *Azotobacter* and the other three to *Azomonas*. Poly-β-hydroxybutyrate accumulated in three of the strains. Unlike most carboxydobacteria, none of these strains could grow using H_2/CO_2.

GRAM-NEGATIVE THERMOPHILIC CARBOXYDOBACTERIA

In our own laboratories we have isolated three strains of a novel obligately aerobic, moderately thermophilic carboxydobacterium (Lyons *et al.*, 1984). The strains were isolated by enrichment culture of sewage (from primary aerobic settlement tanks and from final treated effluent) with 25% CO,

Table 4. Main morphological characteristics of carboxydotrophic bacteria

Strain	Gram reaction	Shape	Spores	Motility	Flagellae and position	Colony colour	G+C ratio	Source
P. carboxydovorans	−ve	Curved rod	No	Most strains	Monotrichous, sub-polar	White/cream	60·7–62·2	Water/sewage
P. carboxydohydrogena	−ve	Rod	No	Yes	Monotrichous, sub-polar	White	58·2	Sewage
P. carboxydoflava	−ve	Rod	No	No	None	Yellow	68·0	River mud
P. compransoris	−ve	Curved rod	No	Yes	Monotrichous, polar	Colourless	63·3	River mud
P. gazotropha	−ve	Tapered rod	No	Yes	Monotrichous, polar	Colourless	66·9	River mud
A. carboxydus	−ve	Rounded rod	No	Yes	Peritrichous	White/cream	63·8	Soil
Arthrobacter 11/×	+ve	Rod/coccus	No	No	None	Colourless	70·2	Beet clamp
P. thermocarboxydovorans	−ve	Tapered rod	No	Most strains	Monotrichous, polar	Yellow/brown	72·0	Sewage
B. schlegelii	Variable	Rod	Yes	Yes	Peritrichous	Cream	63·9–65·4	Lake sediment
Bacillus OMT2	Variable	Rod	Yes	Yes	Peritrichous	Cream	68·6	Lake sediment
Streptomyces G26	+ve	Filamentous	Yes	No	None	Cream/white	ND	Compost

ND: not determined

Table 5. Main physiological characteristics of carboxydotrophic bacteria

					Utilization of:			
Strain	Growth on H_2	Optimum growth temperature	Organic growth factors required	TCA compounds	Sugars	C_1 compounds	Amino acids	Doubling time on CO (h)
P. carboxydovorans	Yes	30	None	Yes	No	No	No	20
P. carboxydohydrogena	Yes	30	None	Yes	Yes	No	Yes	18
P. carboxydoflava	Yes	30	None	Yes	Yes	No	Yes	12
P. compransoris	Yes	30	Thiamine	Yes	No	No	Yes	18
P. gazotropha	Yes	30	Vitamin B_{12}	Yes	No	Methanol	Yes	15
A. carboxydus	No	30	No	Yes	Yes	No	Yes	42
Arthrobacter 11/×	Yes	37	No	Yes	Yes	No	Yes	ND
P. thermocarboxydovorans	No	50	PABA	Yes	No	No	Yes	3
B. schlegelii	Yes	65	None	Yes	No	No	Yes	3
Bacillus OMT2	Yes	65	None	Yes	No	No	Yes	3
Streptomyces G26	Yes	50	None	Yes	Yes	No	Yes	3

ND: not determined

incubated at 45°C or 55°C. The three strains were characterized as Gram-negative oxidase- and catalase-positive rods, with older cultures containing elongated spindle-shaped cells. Although they were isolated from separate enrichment cultures, the three strains were extremely similar; the only major difference between them was the lack of motility shown by one strain. They were therefore considered to represent a single species, *Pseudomonas thermocarboxydovorans* with a DNA G + C content of 72 mole % (*Tables 4* and *5*).

GRAM-POSITIVE MESOPHILIC CARBOXYDOBACTERIA

Hirsch (1961) reported the isolation from lawn soil of Corynebacteria and *Nocardia* species that grew autotrophically with CO. Bartholomew and Alexander (1979) tested pure cultures of known isolates of many different bacteria for the ability to oxidize CO to CO_2 and to fix ^{14}CO into cell material. Only *Nocardia salmonicida* showed appreciable activity with *Nocardia opaca*, a hydrogen-utilizing chemoautotrophic actinomycete fixing labelled CO, but less efficiently. *Arthrobacter* strain 11/x, although isolated as a hydrogen bacterium, was found to grow well with CO (Eberhardt, 1969; Meyer and Schlegel, 1983). Young cultures contained immotile rod-shaped cells which became coccoid in older cultures. Spores were not produced and, as the wall contained LL-diaminopimelic acid and glycine, it was placed in the coryneform bacteria. Although this bacterium was originally isolated at 30°C, the optimum growth temperature was found to be 37°C (*Tables 4* and *5*).

GRAM-POSITIVE THERMOPHILIC CARBOXYDOBACTERIA

Meyer and Schlegel (1983) tested cultures of hydrogen bacteria for the ability to use CO and found that *Bacillus schlegelii* grew well with CO as sole carbon and energy source. Four more strains of carboxydotrophic thermophilic *Bacilli* were isolated from the settling ponds of a sugar factory (Kruger and Meyer, 1984). Three of these strains were similar to *B. schlegelii* and one, designated OMT2, was separated by a higher DNA G + C content and by showing only 59% homology to the other strains in DNA hybridization experiments (Meyer, 1985). The strains produced Gram-variable long rod-shaped cells with terminal spherical endospores which distended the sporangium. Optimum growth temperatures were in the range 65–70°C (*Tables 4* and *5*). We have recently described a Gram-positive thermophilic mycelial bacterium, *Streptomyces* G26, which is moderately thermophilic, with an optimum growth temperature of 50°C (Bell, Williams and Colby, 1985).

GENERAL PROPERTIES OF CARBOXYDOTROPHIC BACTERIA

Although the carboxydobacteria are a taxonomically diverse group of microorganisms they share a number of physiological and biochemical properties. They are all obligately aerobic, oxidase and catalase positive and with

a strictly oxidative metabolism. They contain an inducible CO:acceptor oxidoreductase which oxidizes CO to CO_2, releasing electrons which are used for growth, and fix some of the CO_2 via the ribulosebisphosphate carboxylase cycle. Most species can also grow autotrophically using H_2/CO_2, the exceptions being *P. thermocarboxydovorans*, which does not produce a hydrogenase (EC 1.12.1.2 or 1.12.2.1), *A. carboxydus*, which oxidizes hydrogen but is unable to grow using hydrogen, and the little-studied *Azotobacter* and *Azomonas* strains. Autotrophic growth of the mesophilic strains is slow (*Table 5*) but the thermophilic strains all grow quickly, with doubling times of about 3 hours. All strains are facultatively autotrophic and can grow heterotrophically using a limited range of conventional multicarbon substrates (*Table 5*). Tricarboxylic acid cycle intermediates are used by all carboxydobacteria, but sugars are used only very rarely. Many strains require an organic growth factor (*Table 5*). *P. gazotropha* is the only strain able to use another one-carbon compound for growth, assimilating methanol via the serine cycle (Zavarzin and Nozhevnikova, 1977; Romanova *et al.*, 1978).

Biochemistry of carbon monoxide oxidation in aerobic bacteria

Aerobic carboxydotrophs contain an inducible CO:acceptor oxidoreductase (CO oxidase; CO dehydrogenase), a molybdenum–iron flavoprotein that is responsible for the oxidation of CO to CO_2. In the cell, CO oxidase has several functions: it supplies electrons to the respiratory electron transport chain for the generation of ATP and is also responsible for providing cell carbon as CO_2, and NAD(P)H to drive the reductive CO_2-fixation pentose phosphate (Calvin) cycle. In Gram-negative carboxydotrophs, CO oxidase interacts with the respiratory electron transport chain at the level of ubiquinone or cytochrome *b* and the provision of reduced nicotinamide nucleotides must result from reversed electron flow. Artificial acceptors with E'_o values in the range +0·011 to +0·22 V are generally used in enzyme assays; in our laboratories we routinely use phenazine ethosulphate as the primary electron acceptor (Turner *et al.*, 1984) but thionin (Kim and Hegeman, 1981a) and methylene blue (Meyer and Schlegel, 1980) are also used. The enzymes from the Gram-positive bacteria *Streptomyces* G26 and *Bacillus schlegelii* have been shown to catalyse the reduction of viologen dyes (E'_o = −0·359 to −0·440 V), and *in vivo* reversed electron flow might not be necessary. In all cases, the reaction catalysed is as follows:

$$CO + H_2O + \text{acceptor}_{ox} \rightarrow CO_2 + \text{acceptor}_{red}$$

In vitro, CO oxidase also catalyses the oxidation of NADH, an activity associated with all known molybdenum hydroxylases:

$$NADH + H^+ + \text{acceptor}_{ox} \rightarrow NAD^+ + \text{acceptor}_{red}$$

When CO-grown bacteria are disrupted by sonication or by using a French pressure cell, CO oxidase activity either is equally distributed between the

membrane and soluble fractions (Cypionka, Meyer and Schlegel, 1980) or is found predominantly in the soluble fraction, as with *P. thermocarboxydovorans* and *Streptomyces* G26 in our laboratories, and with *B. schlegelii* (Kruger and Meyer, 1984). *In vivo*, the enzyme is thought to be located on the inner face of the cytoplasmic membrane. This is suggested by the failure of CO oxidases to react with cytoplasmic acceptors such as NAD and has been confirmed by immunolocalization studies using the ferritin and protein A-gold method (Meyer and Rohde, 1984). This work showed that in *P. carboxydovorans* at least, CO oxidase is loosely attached to the cytoplasmic membrane but is released into the cytoplasm at the onset of stationary phase coincidentally with a decrease in the measured CO–O_2 activity in the cells. At this time, however, the CO–methylene blue activity of cell extracts remains unchanged, suggesting that the cytoplasmic form of the enzyme interacts less readily with the respiratory electron transport chain.

CO oxidases from Gram-negative carboxydotrophs

CO oxidases have been purified and characterized from four *Pseudomonas* spp.: *P. carboxydovorans, P. carboxydoflava, P. carboxydohydrogena* and *P. thermocarboxydovorans* (Meyer and Schlegel, 1980; Kim and Hegeman, 1981a; Meyer, 1982; Meyer and Rajagopalan, 1984; Turner *et al.*, 1984). The first three are mesophilic strains while the latter is a moderate thermophile. The CO oxidases from the mesophilic carboxydotrophs are identical in all measurable parameters (Meyer and Rajagopalan, 1984) and have been shown to have some measure of immunological identity (Meyer and Schlegel, 1983), whereas the analogous enzyme from *P. thermocarboxydovorans* does show significant differences, principally in respect of its temperature stability and affinity for the reducing substrate.

KINETIC PROPERTIES

The properties of CO oxidases are summarized in *Table 6*. The enzymes are notably specific with respect to their reducing substrates, none of the enzymes having been shown to oxidize any compound other than CO (with the exception of NADH). The affinities of the mesophilic enzymes for CO are similar (K_m about 53–63 μM; Meyer and Schlegel, 1980; Kim and Hegeman, 1981a) whereas the affinity of the enzyme from *P. thermocarboxydovorans* for its substrate is 100-fold greater (K_m about 0·6 μM; Turner *et al.*, 1984)—a very significant difference which might be related to the higher growth temperature of the organism; CO is considerably less soluble at higher temperatures. This latter enzyme also differs from the others in its much higher optimum temperature as measured using the usual spectrophotometric assay. Although thermal denaturation is rapid at this temperature, the higher optimum for this enzyme does reflect a greater tolerance to high temperatures.

The enzymes from all sources are able to use a variety of electron acceptors *in vitro* provided that their E'_0 values lie in the range +0·011 V to +0·429 V. Although there are minor divergences reported between the electron acceptor ranges of the CO oxidases from different Gram-negative sources, these may well reflect variations in experimental technique and conditions rather than any fundamental differences in the enzymes themselves.

MOLECULAR WEIGHT AND SUBUNIT COMPOSITION

Reported molecular weight values for CO oxidases from the Gram-negative carboxydotrophs have varied from 170 000 (Cypionka, Meyer and Schlegel, 1980) to 400 000 (Kim and Hegeman, 1981a) with the values obtained depending on (a) the organism used, (b) the method of determination, and (c) the workers involved. However, the situation has now resolved itself to the extent that the following statements can be made with confidence:

1. The enzymes from *P. carboxydovorans*, *P. carboxydoflava* and *P. carboxydohydrogena* have the same molecular weight (Meyer and Rajagopalan, 1984);
2. Gel-permeation chromatography (whether on soft gels or HPLC sizing columns) yields molecular weights that are higher than the values given by centrifugation methods (Meyer and Rohde, 1984; Turner *et al.*, 1984);
3. The best estimates yield values for the enzymes from all four sources that lie in the range 230 000–300 000;
4. The enzyme from *A. carboxydus* appears to be smaller.

With such a complex protein as CO oxidase, it is not surprising that different determination methods yield different results. It must also be borne in mind that CO oxidase is a membrane protein and, as isolated, may have other membrane components associated with it. It has been stated that molecular weight values obtained from HPLC sizing columns are the most reliable (Meyer and Rohde, 1984) but there seems to be no *a priori* reason why this should be so.

The subunit composition of the CO oxidases in this group is not clear. Three different subunits have been described on dissociation of the enzymes from *P. carboxydohydrogena* and *P. carboxydovorans* on heating with 1% SDS for at least 1 min (Kim, Kirkconnell and Hegeman, 1982; Meyer and Rohde, 1984). Their molecular weights are 14–17 000 (S), 28–34 000 (M), and 85–86 000 (L). Less rigorous treatment of the enzyme from *P. carboxydovorans* (heating for less than 1 min at 90°C, or treatment on ice, or at room temperature) produced only high molecular weight subunits of *c.* 90 000 molecular weight which retained the brown colour of the native enzyme. The absorption spectrum of the treated material indicated that the iron–sulphur centres were intact. These large subunits also retained CO–methylene blue and NADH–methylene blue activity as detected by activity staining of the gels. Reduction with mercaptoethanol was not necess-

ary for dissociation into either three subunits or, under the milder conditions, into large subunits only, suggesting that the subunits are not covalently attached. Meyer and Rohde (1984) concluded that the most likely configuration in the intact enzyme is L_3, with this structure breaking down on harsh treatment to a $L_2M_2S_{1-2}$ configuration.

METAL AND COFACTOR COMPOSITION

The absorption spectra of the CO oxidases from the three Gram-negative carboxydotrophs, *P. carboxydovorans*, *P. carboxydoflava* and *P. carboxydohydrogena*, are identical with peaks at 280 nm, 420–450 nm and broad shoulders at 390 nm and 550 nm (Meyer, 1982; Meyer and Rohde, 1984). Purified CO oxidase from *P. thermocarboxydovorans* showed maxima at 270, 335 and 420 nm with a distinct shoulder at 460 nm (Turner *et al.*, 1984). In the latter case, boiling the enzyme with 1% SDS caused a change in the absorption spectrum to that typical of free flavin. The enzymes from all four micro-organisms have been shown to contain 2 FAD, 8 Fe, 8 acid-labile sulphide and 0·7–0·9 Mo per mole of enzyme, with copper (0·7–1·54 atoms/mole) and zinc (2–3 atoms/mole) also being present in most preparations.

CO oxidase from Gram-positive bacteria

Two classes of Gram-positive aerobic carboxydotrophic bacteria are known. Bell, Williams and Colby (1985) described a thermophilic mycelial carboxydotroph, designated *Streptomyces* G26, isolated from compost and growing optimally at 50°C. Kruger and Meyer (1984) reported the isolation of four strains of *B. schlegelii*, a thermophile able to grow on CO with an optimum temperature of 70°C. Although this organism is described as Gram-variable, its allocation to the largely Gram-positive genus *Bacillus* justifies its inclusion here.

STREPTOMYCES G26

The partial purification of the CO oxidase from this organism has been described, together with some properties of the partially purified enzyme (Bell, Williams and Colby, 1985). The most surprising finding was the ability of the enzyme to reduce low-potential acceptors such as methyl- and benzyl-viologens at rates comparable with its activity with the more usual acceptors such as phenazine ethosulphate and methylene blue. This CO oxidase has now been purified to homogeneity, largely by using HPLC techniques, and its properties investigated (*Table 6*). Despite its activity with viologens, the enzyme is clearly an iron–sulphur/FAD/molybdenum hydroxylase similar in most respects (molecular weight, cofactor composition, kinetic properties) to the analogous enzyme from the Gram-negative carboxydotrophs. Like the CO oxidase from the Gram-negative thermophile *P. thermocarboxydovorans*, this enzyme has an unusually low K_m for CO.

Table 6. Properties of CO oxidases from Gram-negative and Gram-positive carboxydotrophs

	Gram-negative carboxydotrophs		Gram-positive carboxydotrophs	
Properties	*Pseudomonas carboxydovorans* *Pseudomonas carboxydohydrogena* *Pseudomonas carboxydoflava*	*Pseudomonas thermocarboxydovorans*	*Streptomyces* G26	*Bacillus schlegelii*
Analytical data				
Molecular weight	300 000	270 000	280 000	300 000
Subunits	90 000	100 000	109 000	ND
Configuration	trimer(?)	trimer(?)	trimer(?)	ND
Metal/Sulphide	8Fe:8S:0·9Mo	8Fe:8S:0·8Mo	8Fe:8S:?Mo	ND
Flavin	2FAD	2FAD	2flavin	ND
Molybdenopterin	present	ND	ND	ND
Nickel	absent	absent	absent	ND
Kinetic data				
Optimum pH	7·0	7·5	7·2	ND
Optimum temperature (°C)	63	80	65	95
K_m for CO (μM)	53	0·6	1·3	ND
Acceptors with positive E'_o reduced	yes	yes	yes	yes
Viologens reduced	no	no	rapidly	slowly
Specific activity of purified enzyme (U/mg protein)	3·9	11·6	14·0	ND

ND: not determined

In the Gram-negative mesophilic carboxydotrophs, where CO oxidase has been shown to interact with the electron transport chain via ubiquinone or cytochrome *b*, NAD(P)H provision for biosynthesis is assumed to arise from reversed electron flow. In *Streptomyces* G26, electrons from reduced CO oxidase may enter the electron transport chain via acceptors such as rubredoxin or ferredoxin and reduce NAD(P) directly without the expenditure of metabolic energy. Further work on the mechanism of electron transport in this organism is required before the *in vivo* significance of the broad electron-acceptor specificity of this CO oxidase can be fully assessed.

BACILLUS SCHLEGELII

The CO oxidase from this Gram-positive carboxydotroph has been studied in crude bacterial extracts only (Kruger and Meyer, 1984). Between 70% and 90% of the enzyme activity (depending on strain) was found in the soluble extract following cell breakage and ultracentrifugation. Growth of the organism on CO was dependent on molybdenum, suggesting that this CO oxidase is also a molybdenum hydroxylase, and its molecular weight was found to be similar to that of the enzyme from the Gram-negative mesophiles such as *P. carboxydovorans*. CO oxidase from crude soluble extracts also reduced methyl-viologen although in this case the activity with the low-potential acceptor was low compared with its activity with methylene blue. The properties of the *B. schlegelii* CO oxidase are summarized in *Table 6*.

Mechanism of action of CO oxidase

IRON–SULPHUR CLUSTERS AND FAD AS REDOX CENTRES

Air-oxidized CO oxidase from *P. carboxydovorans* is brown with overlapping absorption bands throughout the visible region below 600 nm and with maxima at 420 nm and 450 nm and shoulders at 390 nm and 550 nm (Meyer, 1982). On reduction with dithionite, the absorption in the 420–450 nm and 500–600 nm regions was markedly reduced, indicating that both the FAD and iron–sulphur (Fe–S) centres are reduced. On treatment with CO alone under strictly anaerobic conditions, the enzyme was partially reduced (35% of FAD, 39% of Fe–S) but in the presence of methylene blue the enzyme was completely reduced. This may indicate the necessity for a concerted reaction between enzyme, reducing substrate and electron acceptor. Complete but slow reduction of the enzyme could also be achieved by photoreduction in the presence of 20 mM EDTA and 2·4 M urea. On admitting air gradually, the reoxidation of the enzyme could be followed by an increase in absorption in the FAD and Fe–S regions: reoxidation occurred simultaneously in both. No flavin semiquinone formation was observed at any stage during oxidation or reduction of CO oxidase.

Electron paramagnetic resonance (EPR) studies on the CO oxidases from *P. carboxydovorans* and *P. carboxydohydrogena* (Bray *et al.*, 1983) revealed the presence of two different signals corresponding to Fe–S centres on

reduction with either dithionite or CO and methylene blue. Both signals (designated Fe–S 1 and Fe–S 2) were present at 16K but at higher temperatures (*c.* 49K) only the Fe–S 1 signal was developed. Further analysis of the signals demonstrated that the structures and chemical environments of the Fe–S centres in CO oxidase are similar to those of xanthine oxidase, as is the calculated distance (0·8–1·5 nm) between the Mo(V) and Fe–S 1 centres. The active-site structures of these quite different enzymes therefore seem similar. A weak flavin free-radical signal was also observed on reduction of CO oxidase with CO or with dithionite.

EVIDENCE FOR THE STRUCTURE AND ROLE OF THE MOLYBDENUM COFACTOR

Molybdopterin is the molybdenum cofactor in all molybdenoenzymes so far examined, with the exception of nitrogenase. The presence of this cofactor in the CO oxidase from *P. carboxydovorans* was confirmed by the ability of material released from the enzyme by treatment with heat (90°C for 30 s), acid, 1% SDS or 6M guanidine hydrochloride, to complement NADPH–nitrate reductase (EC 1.6.6.1) activity in extracts of *Neurospora crassa* nit-1 mutant (Meyer and Rajagopalan, 1984). The proposed structure of molybdopterin is shown in *Figure 1* (Johnson and Rajagopalan, 1982). Molybdopterin is tightly but non-covalently bound to CO oxidase and, as with sulphite oxidase (EC 1.8.3.1), xanthine oxidase (EC 1.1.3.22) and xanthine dehydrogenase (EC 1.1.1.204), the enzyme contains two molecules of the cofactor.

Electron paramagnetic resonance (EPR) spectroscopy on untreated CO oxidase from *P. carboxydovorans* or *P. carboxydohydrogena* (Bray *et al.*, 1983) revealed a Mo(V) resting signal. However, evidence for a catalytic role for Mo was not obtained as direct interaction of the resting Mo(V) form of the enzyme with CO was not demonstrable as an isotopic effect on incubating the enzyme with ^{13}CO. Evidence for a crucial catalytic role for molybdenum in CO oxidase, however, includes: (1) inhibition of enzyme activity by methanol which traps Mo in the pentavalent state (Meyer, 1982);

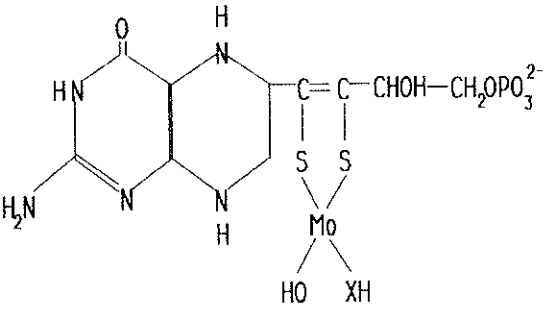

Figure 1. Proposed structure of molybdopterin. From Johnson and Rajagopalan, 1982.

(2) dependence of growth on CO, but not heterotrophic growth, on the presence of Mo in the growth medium (Kalnowski, 1980; Meyer and Schlegel, 1983); (3) inhibition of CO-autotrophic growth by the molybdenum antagonist, tungstate (Kalnowski, 1980).

IMPORTANCE OF ARGININE IN CO BINDING

In our laboratories, we have investigated the catalytic role, if any, of certain groups at the active centre of the CO oxidase from *P. thermocarboxydovorans*, by using active site-directed reagents (A. Halder, personal communication). Time-dependent inhibition by modifying reagents and protection by substrate can be taken as evidence for a particular residue to which the reagent binds being at or near the active site. Neither carbonyl-binding reagents, such as semicarbazide and hydroxylamine, nor chelating agents inhibit CO oxidase, nor in most cases do sulphydryl-binding reagents such as ethylmaleimide. Iodoacetate does inhibit, but this is probably due to the alkylation of other moieties such as FAD. Both butanedione and phenylglyoxal specifically modify arginine under mild conditions and have been used to investigate the role of arginine in several enzymes. Both these reagents inhibit CO oxidase in a time-dependent manner and non-linear semilogarithmic plots (of ln V_i/V_o against time) are observed. Protection against inhibition by either reagent is observed on incubating the enzyme with the inhibitor in the presence of CO. These results indicate the probable presence of arginine at the CO-binding site (A. Halder, personal communication).

POSSIBLE INVOLVEMENT OF SELENIUM

Meyer and Rajagopalan (1984) have demonstrated with the soluble CO oxidase from *P. carboxydovorans*, a 6- to 8-fold activation of the enzyme on aerobic incubation with selenite. Both the CO–methylene blue and the CO–2,6-dichlorophenol-indophenol activities were enhanced, whereas the NADH–methylene blue activity, also demonstrated by the soluble enzyme, was unaffected. Fully activated enzyme contains Se, Mo and FAD in a 1:1:1 ratio. Treatment with [^{75}Se]-selenite indicated that the selenite was covalently incorporated into the enzyme, probably after the sulphurs of half-cystine residues as follows:

$$4RSH + H_2SeO_3 \rightarrow RSSeR + RSSR + 3H_2O$$

Bound selenium was not found to be associated with the molybdopterin prosthetic group because denaturing agents that release the Mo cofactor fail to remove bound selenium. The physiological significance of this observation is doubtful in view of the failure of selenite to activate the membrane-bound form of the enzyme.

Electron transport from CO oxidase

Electron transport in the aerobic carboxydotrophic bacteria has been studied in only a few representatives of the Gram-negative mesophiles, and in detail only in *P. carboxydovorans*. Carboxydotrophs have been shown to contain normal electron transport components, including *a*-, *b*- and *c*-type cytochromes at concentrations comparable with those found in other aerobic bacteria (Cypionka and Meyer, 1983). *P. carboxydovorans* contains ubiquinone (UQ_{10}) as the only quinone (Meyer and Schlegel, 1980). Cell extracts of *P. carboxydohydrogena* that had lost their CO-oxidizing activity following UV treatment showed a restoration of activity on adding back UQ_{10}, suggesting that this component might be the physiological acceptor for CO oxidase (Kim and Hegeman, 1981b). Early work also established that the respiratory electron transport chains of *P. carboxydovorans* and *P. carboxydohydrogena* are branched with more than one terminal oxidase. Nevertheless, carboxydotrophs differ from most aerobes in the insensitivity of their growth and respiration to poisoning by CO. This resistance to poisoning by CO is constitutive and is therefore not due to the presence of CO oxidase at the cell membrane. Recent work on *P. carboxydovorans*, involving the use of electron transport inhibitors, room- and low-temperature spectroscopy and photochemical CO action spectra (Cypionka and Meyer, 1983), has established in *P. carboxydovorans* the existence of heterotrophic (used during the oxidation of NADH or succinate) and autotrophic (used during the oxidation of H_2 or CO) branches to the electron transport chain, with the autotrophic branch being completely insensitive to CO.

The following evidence favoured this conclusion:

1. NADH oxidation was 20- to 90-fold more sensitive to antimycin A than the oxidation of CO or H_2;
2. Oxidation of CO or H_2 was strongly sensitive to HQNO (2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide), whereas NADH was only partially sensitive;
3. During steady-state oxidation of CO or H_2 the *b*-type cytochromes were reduced to a greater extent than during the oxidation of all other substrates;
4. Even after long incubation with CO or H_2 the *b*-type cytochromes were more reduced than after incubation with other substrates.

The authors concluded that *b*-type cytochromes are preferentially used by electrons originating from CO or H_2 (autotrophic branch) whereas NADH is preferentially oxidized via cytochromes *c* and *a*. Further evidence for a branched chain was: (1) oxidation of NADH or H_2 was only partially inhibited by CO; (2) biphasic titration curves with cyanide indicated two terminal oxidases. Evidence from inhibitor studies favoured the branching point occurring at the level of UQ_{10} or *b*-type cytochrome and low-temperature spectra with NADH or H_2 indicated the involvement of cytochromes $b_{558(LT)}$ and $b_{561(LT)}$ in the autotrophic branch, and of cytochrome $b_{556(LT)}$

in the heterotrophic branch. Cytochrome a_1 was identified as the terminal oxidase of the heterotrophic branch by the photochemical CO action spectrum of the tetramethyl-*para*-phenylenediamine (TMPD) oxidase activity. The terminal oxidase of the autotrophic branch could not be identified in this way due to its insensitivity to CO. The following evidence, however, favoured cytochrome *o* ($=b_{563}$) as having this function:

1. H_2O_2 was not the end product of the autotrophic branch;
2. Cytochromes *d* (a_2) and the alternative oxidase found in many eukaryotes was absent;
3. CO difference spectra revealed a maximum at 416 nm and troughs at 433 nm and 563 nm, indicative of cytochrome *o*;
4. Cytochrome *o* was the first to react with oxygen during the reoxidation of reduced extracts and was the least reducible cytochrome.

As a result of their elegant study, Cypionka and Meyer (1983) formulated a model for electron transport in *P. carboxydovorans* as shown in *Figure 2*.

ANAEROBIC BACTERIA

Anaerobic bacteria capable of transforming CO are taxonomically diverse. They include the obligate anaerobes (methanogenic, acetogenic and sul-

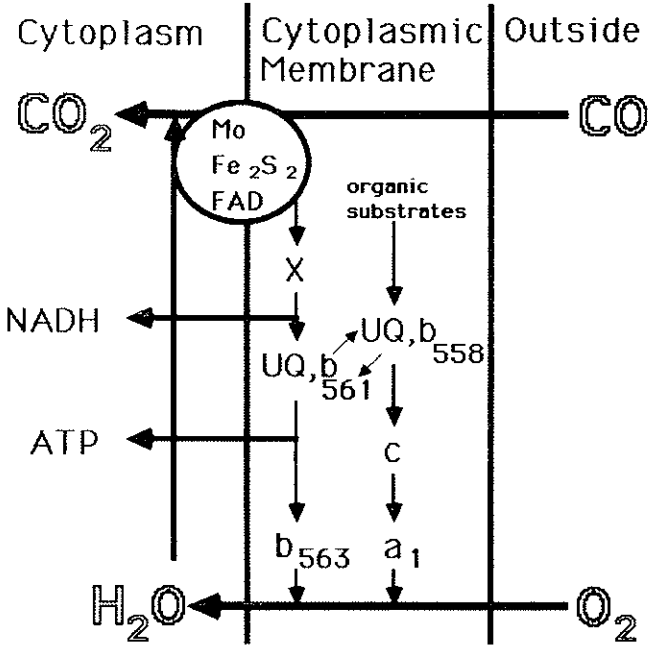

Figure 2. Function and localization of CO oxidase in *Pseudomonas carboxydovorans* and its interaction with the CO-insensitive branch of the respiratory chain. Redrawn from Meyer (1985).

phidogenic bacteria) and the facultative anaerobic photosynthetic bacteria (*Table 2*). The consumption of CO by many of these species is considered to be non-utilitarian. The enzyme associated with the oxidation of CO in the obligate anaerobes, CO dehydrogenase, is produced constitutively by many species, and is stimulated by the presence of substrates other than CO. It is likely that many more species will be described which can utilize CO, as this property has not been extensively examined in nature. Of the CO-utilizing species which have been described, most can also utilize H_2/CO_2. However, nearly all required prolonged periods of adaptation to CO (Genthner and Bryant, 1982; Lynd, Kerby and Zeikus, 1982; Kerby and Zeikus, 1983; Zeikus, 1983; O'Brien *et al.*, 1984) and the resulting CO-utilizing strains are often considered to be metabolic mutants (Krzycki, Wolkin and Zeikus, 1982; Lynd, Kerby and Zeikus, 1982).

The production of CO has been discussed in previous sections but its entry into anaerobic environments is poorly understood. It has been known for some time that *Streptococcus mitis* could form CO from the degradation of haem or hydroxycobalamin (Engel *et al.*, 1972, 1973), though in both instances only in the presence of air. The first report of CO-formation under strictly anaerobic conditions was the isotopic exchange between $^{14}CO_2$ and CO in cell suspensions of *Desulfovibrio desulfuricans*, supplemented with reduced methyl viologen (Yagi and Tamiya, 1962). This isotopic exchange has more recently been reported in *Clostridium thermoaceticum* between the carboxy group of acetyl-CoA and CO (Hu, Drake and Wood, 1982) and in *Butyribacterium methylotrophicum* (Lynd, Kerby and Zeikus, 1982; Zeikus, 1983). Production of CO has also been observed by cultures of *Methanobacterium thermoautotrophicum* grown on H_2/CO_2 (Conrad and Thauer, 1983; Eikmanns and Thauer, 1984; Eikmanns, Fuchs and Thauer, 1985) and is related to the assimilation of CO via its CO dehydrogenase. This relationship between CO production and CO dehydrogenase activity has also been shown in *Acetobacterium woodii* and *C. thermoaceticum* (Diekert, Hansch and Conrad, 1984).

Carbon monoxide-oxidizing activity in whole cells and crude cell-free extracts

METHANOGENIC BACTERIA

The first indication that anaerobic bacteria could metabolize CO was the observation that CO was converted to methane and CO_2 by sewage sludge, and by mixed cultures of methanogens (Fischer, Lieske and Winzer, 1931, 1932). Kluyver and Schnellen (1947) demonstrated the production of methane and CO_2 from CO using cell suspensions of *Methanosarcina barkeri* and *Methanobacterium formicicum*. Daniels *et al.* (1977) isolated several other methanogens capable of reducing CO with H_2 to methane according to the equation:

$$CO + 3H_2 \rightarrow CH_4 + H_2O \ (G'_o = -150 \text{ kJ/mol CO})$$

They also showed that the methanogen *Methanobacterium thermoautotrophicum* could be adapted to grow with CO as its sole source of carbon and energy. It could grow with up to 30% CO in the headspace gas, but was completely inhibited by concentrations greater than 60%. The maximum growth rate was only 1% of that on H_2/CO_2. Athough *M. thermoautotrophicum* has a doubling time on H_2/CO_2 of 60 minutes at 65°C (Zeikus, 1983), the doubling time on CO was approximately 100 hours. Cell-free extracts of *M. thermoautotrophicum* catalysed the rapid reduction of F_{420} with CO. Coenzyme F_{420} is a low-potential electron carrier mediating between hydrogenase and $NADP^+$ in methanogenic bacteria. Growth of this organism is nickel-dependent and at low nickel concentrations ($NiCl_2$ < 100 nM) the amount of cells formed was roughly proportional to the amount of nickel added (Schonheit, Moll and Thauer, 1979).

Daniels *et al.* (1977) also showed that *M. barkeri* oxidized small amounts of CO added during growth on H_2/CO_2, and that this was associated with a methyl viologen-linked CO dehydrogenase activity in the cell extracts. Using a prolonged period of cultural adaptation and sequential transfers of culture in medium that contained 50 mM methanol under a 100% CO gas atmosphere, followed by transfers of the culture under CO alone, a CO-adapted strain of *M. barkeri* was isolated (O'Brien *et al.*, 1984). Its doubling time under CO alone was about 65 hours in the most rapid phase of growth (O'Brien *et al.*, 1984). The CO dehydrogenase of *M. barkeri* is constitutive and very active in acetate-grown cells where it constitutes up to 5% of the soluble cellular protein (Krzycki, Wolkin and Zeikus, 1982; Krzycki and Zeikus, 1984a), approximately five times higher than in H_2/CO_2- or CO-grown cells. Its affinity for CO is very low (K_m of 5 mM for CO) which suggests that its primary function is not the consumption of environmental CO (Krzycki and Zeikus, 1984a). Soluble cell-free extracts of *M. barkeri* were capable of transforming acetate to methane in the presence of ATP and H_2 (Krzycki and Zeikus, 1984b). The mechanism of conversion of acetate to methane involves the CH_3– group of acetate being the direct precursor of methane:

$$^1CH_3{}^2COOH \rightarrow {}^1CH_4 + {}^2CO_2$$

The direct involvement of CO dehydrogenase in this process was shown by inhibition of acetate-dependent methanogenesis by addition of 15 μM KCN or purified anti-CO-dehydrogenase-IgG to crude cell extracts (Krzycki, Lehman and Zeikus, 1985).

CO dehydrogenase activity has also been detected in many other methanogens (Daniels *et al.*, 1977). Its role in the fixation of CO_2 has been studied in *M. thermoautotrophicum* (Stupperich *et al.*, 1983; Stupperich and Fuchs, 1983, 1984a,b) and in *Methanobrevibacter arboriphilicus* (Hammel *et al.*, 1984), and in acetate metabolism in *M. barkeri* (Kenealy and Zeikus, 1982; Krzycki, Wolkin and Zeikus, 1982; Stupperich *et al.*, 1983; Zeikus, 1983), *Methanothrix soehngenii*, a methanogen which is able to grow exclusively on acetate (Kohler and Zehnder, 1984) and *Methanosarcina* spp. (Nelson and

Ferry, 1984). *M. thermoautotrophicum* grown in the presence of ^{14}CO showed incorporation of radioactivity specifically into metabolic intermediates derived from acetyl-CoA (Stupperich *et al.*, 1983). These results are compatible with the proposed function of CO dehydrogenase in carbonylation of a methyl group during acetyl-CoA/acetate synthesis in both acetogenic (Hu, Drake and Wood, 1982) and methanogenic bacteria (Kenealy and Zeikus, 1982; Stupperich *et al.*, 1983). *Methanothrix soehngenii* grows exclusively by the assimilation of acetate (Huser, Wurhman and Zehnder, 1982). The finding of high levels of CO dehydrogenase in this organism suggests a physiological role in the acetoclastic reaction, rather than in fixation of CO_2 to form cell material (Kohler and Zehnder, 1984).

HOMOACETOGENIC BACTERIA

There are two pathways for the total synthesis of acetate from CO_2—the methyl tetrahydrofolate pathway and the serine pathway (Diekert, Fuchs and Thauer, 1985). CO dehydrogenase activity has been detected only in those acetogenic bacteria with the methyl tetrahydrofolate pathway (Diekert and Thauer, 1978). Many species of acetogenic bacteria have been shown to contain a protein with CO dehydrogenase activity. These include *Clostridium pasteurianum* (Fuchs, Schnitker and Thauer, 1974; Thauer *et al.*, 1974a), *Clostridium thermoaceticum, Clostridium formicoaceticum* (Diekert and Thauer, 1978, 1980), *Clostridium thermoautotrophicum* (Wiegel, 1982), *Butyribacterium methylotrophicum* (Lynd, Kerby and Zeikus, 1982) and *Acetobacterium woodii* (Deikert and Ritter, 1983a; Ragsdale, Ljungdahl and DerVartanian, 1983a). All of these species are also able to synthesize acetate from H_2/CO_2 (Barker and Kamen, 1945; Andreesen, Gottschalk and Schlegel, 1970; Balch *et al.*, 1977; Zeikus *et al.*, 980; Genthner, Davis and Bryant, 1981).

Thauer *et al.* (1974a), while studying hydrogenase in cell-free extracts of glucose-grown *C. pasteurianum*, discovered that the extracts also catalysed the reduction of methyl viologen with CO. In a second paper (Thauer *et al.*, 1974b), this property was investigated by culturing *C. pasteurianum* on glucose in a mineral medium in the absence of CO. Crude cell-free extracts of the culture catalysed the reduction of FAD, FMN, methyl and benzyl viologens and methylene blue on addition of CO; $NAD(P)^+$ and *C. pasteurianum* ferredoxin were not effective as electron acceptors. The reduction of 1·95 moles of the one-electron accepting dye, methyl viologen, per mole of CO added to the reaction mixture, was taken to indicate that the oxidation product of CO was CO_2. Exposure of the extract to oxygen led to total loss of activity. Activity was also lost during incubation with cyanide (10 μM), but the inactivation was prevented and apparently reversed in the presence of CO. Incubation of cell extracts with methyl iodide (2·5 mM) in the dark decreased the CO-oxidizing activity; this process was accelerated in the presence of CO. Extracts inactivated in the presence of alkyl halides could be photo-reactivated. These findings led to the suggestion that the CO-oxidizing activity may be a cobalt-containing corrinoid protein (Thauer *et al.* (1974a).

CO dehydrogenase activity was not found in all species of *Clostridium* (Diekert and Thauer, 1978). *Clostridium aceticum* and *Clostridium perfringens* (Kluyver and Schnellen, 1947; Stephenson, 1949) oxidized CO through an exchange or non-fortuitous reaction. *Clostridium aceticum* produced acetate from H_2/CO_2 (Braun, Mayer and Gottschalk, 1981) but there is no evidence for the existence of a CO dehydrogenase or for its role in anabolism or catabolism. *C. acid-urici* and *C. cylindrosporum* did not contain CO-oxidizing activity when grown on hypoxanthine, but synthesized acetate via the corrinoid-independent serine pathway. *C. formicoaceticum* and *C. thermoaceticum*, grown on fructose and glucose respectively, synthesized acetate via a methyl tetrahydrofolate-dependent pathway and contained CO-oxidizing activity. However, the mechanism for coupling of CO oxidation with reduction of CO_2 to acetate was not fully understood. The authors suggested that the ability of the enzyme to react with CO indicated that the prosthetic group contains a transition metal, and the ability to reduce methyl viologen and triquat ($E'^{o} = -548$ mV) suggested that the enzyme must have an electron-accepting group of very low redox potential. Kinetic plots of activity with CO and methyl viologen were typical of a 'ping-pong' mechanism, suggesting that the enzyme may occur in an oxidized or reduced form.

Diekert, Graf and Thauer (1979), when growing *C. pasteurianum* in glucose–ammonium medium with iron as sole transition metal, found that the production of the enzyme, CO dehydrogenase, was dependent solely on the addition of nickel to the medium. Diekert and Thauer (1980) also showed that nickel uptake paralleled the synthesis of CO dehydrogenase activity in *C. thermoaceticum* and *C. formicoaceticum*. Co-migration of CO dehydrogenase and ^{63}Ni activity was also demonstrated during gel electrophoresis of a crude extract of *C. pasteurianum* grown in the presence of ^{63}Ni. This organism is a nitrogen fixer and butyric acid fermenter and was not known to synthesize acetate from one-carbon compounds (Drake, 1982). Co-migration of CO dehydrogenase activity and ^{63}Ni activity was observed during electrophoresis at pH 9·3 or pH 7·7. Incubation with oxygen for 30 min led to the inactivation of the enzyme in crude cell extracts and to the dissociation of the ^{63}Ni-containing component from the enzyme, as determined by gel electrophoresis. The structure of the ^{63}Ni-containing component was not clear.

The Marburg strain of *B. methylotrophicum* contained CO dehydrogenase and consumed CO during growth on a variety of substrates in the presence of 100% CO in the gas phase (Lynd, Kerby and Zeikus, 1982). However, by a prolonged selection procedure, requiring five transfers of culture over 12 weeks, a strain of *B. methylotrophicum* was isolated which grew with 100% CO as sole source of carbon and energy. The strain had a doubling time of 14 hours on CO compared with a doubling time on H_2/CO_2 of 9 hours (Lynd, Kerby and Zeikus, 1982). The ability of the strain to grow on CO was stable through multiple transfers in the absence of CO, indicating that the difference between the CO strain and the parent, Marburg strain, was due to a genetic, mutational event. The only property found to be unusual to the CO strain, apart from growth on CO, was the inhibition of this growth by formate. There were quantitative differences in enzyme levels

between the two strains. In particular, CO dehydrogenase in the Marburg strain was higher when grown on methanol-acetate-CO_2 than when the CO strain was grown on CO, inferring that more than CO dehydrogenase activity is required for growth on CO. The mixotrophic (i.e. growing on two or more substrates simultaneously) metabolism of *B. methylotrophicum* differs from that in other acetogens and methanogens, in that CO-utilization contributes to cell synthesis, and CO toxicity is not observed (Lynd, Kerby and Zeikus, 1982). The end products of either H_2/CO_2 or CO fermentation were acetate and CO_2, with traces of butyrate (Lynd and Zeikus, 1983).

Eubacterium limosum is capable of growth with CO as sole energy source (Genthner and Bryant, 1982). Unlike the methanogens and other acetogens which can use CO, this species did not require a prolonged period of adaptation. The organism had a doubling time of 7 hours with up to 50% CO—approximately the same as when grown with H_2/CO_2 or methanol. Growth was inhibited at concentrations of CO above 50%, but slight growth occurred up to 75% CO, the highest concentration tested. It is therefore more tolerant of CO than *M. thermoautotrophicum* which was inhibited above 30% CO, but is similar to *M. barkeri* which has an optimum at 50% CO. The growth of *E. limosum* is approximately ten times faster than any of the methanogens. The end products of either H_2/CO_2 or CO fermentation were acetate with traces of butyrate (Genthner, Davis and Bryant, 1981). Using the techniques applied to *B. methylotrophicum*, Kerby and Zeikus (1983) isolated a strain of *C. thermoaceticum* capable of growth on either an atmosphere of H_2/CO_2 or CO (100%) alone. The doubling times at 55°C on H_2/CO_2 or CO as energy source were 18 hours and 16 hours respectively. *A. woodii* (Diekert and Ritter, 1983a; Ragsdale, Ljungdahl and DerVartanian, 1983a) and *C. thermoautotrophicum* (Wiegel, Braun and Gottschalk, 1982) have also been culturally adapted to grow with CO as sole energy source (Zeikus, 1983).

Two further genera of anaerobic bacteria can oxidize CO but the mechanisms involved are quite different from those in acetogenic and methanogenic bacteria.

SULPHATE-REDUCING BACTERIA

Yagi (1958) demonstrated the conversion of CO to CO_2 by cell-free extracts of *Desulfovibrio desulfuricans*. In the presence of sulphite as an oxidant and using Warburg manometric methods, he deduced a reaction equation:

$$H_2SO_4 + 3CO \rightarrow 3CO_2 + H_2S$$

Addition of methyl viologen ($E'^{\circ} = -440$ mV) as electron acceptor, increased the reaction rate. Using radioactive tracer methods with $^{14}CO_2$, and omitting sulphite from the reaction mixture, a new mechanism was deduced:

$$^{14}CO + H_2O \rightarrow {}^{14}CO_2 + H_2$$

No activity was observed with boiled extract or when CO was replaced by N_2. Formate was not an intermediate. There is, however, no evidence that *Desulfovibrio* utilizes CO for growth (Yagi, 1959). It is concluded that there are two enzymes involved in the overall reaction—a CO-activating enzyme and a hydrogenase, using water as an oxidant. This reaction is reversible but little is known of its physiological role in the intact cell (Yagi and Tamiya, 1962).

PHOTOSYNTHETIC BACTERIA

Hirsch (1968) reported the growth of *Rhodopseudomonas* spp. at the expense of CO under anaerobic photosynthetic conditions. Carbon monoxide was used as the major carbon source and water was cleaved to provide hydrogen as the source of reducing power. Other non-sulphur purple bacteria, *Rhodopseudomonas gelatinosa* (Uffen, 1975, 1976; Dashekivicz and Uffen, 1979) and *Rhodopseudomonas rubrum* (Uffen, 1981) can also grow with CO, but in the dark. Doubling times of 6–7 h have been reported (Uffen, 1976, 1981, 1983). Hydrogen is generated to provide the reducing power for the fixation of the CO_2 produced from CO. The CO-oxidative activity in *R. gelatinosa* strain 1 is membrane associated and appears to operate as a principal anaerobic, respiratory energy-yielding reaction in ATP regeneration (Wakim and Uffen, 1983). It is only in the above strains that CO-oxidizing activity is induced in the presence of CO.

Function of the CO dehydrogenase in methanogens and acetogens

It is unlikely that CO is a natural substrate for growth of either methanogens or acetogens. This is exemplified by their poor growth with CO or the need for prolonged adaptation to the substrate (Daniels *et al.*, 1977; Lynd, Kerby and Zeikus, 1982; Genthner and Bryant, 1982; Kerby and Zeikus, 1983) although it does appear to be an intermediate in the metabolism of chemotrophic anaerobes.

In methanogens, CO dehydrogenase has been proposed to function in the synthesis of a carboxyl intermediate from CO_2 or CO and possibly in the carboxylation of a methyl intermediate in the synthesis of acetyl CoA in both acetogenic (Hu, Drake and Wood, 1982; Eden and Fuchs, 1982; Diekert and Ritter, 1983b; Kerby, Niemczura and Zeikus, 1983; Ragsdale *et al.*, 1983) and methanogenic bacteria (Kenealy and Zeikus, 1982; Stupperich *et al.*, 1983). In methanogens, CO dehydrogenase has both an anabolic and a catabolic role. The anabolic role is similar to that described for the acetogens; that is the synthesis of acetyl CoA from one-carbon substrates by the carboxylation of a methyl group (Kenealy and Zeikus, 1982; Stupperich *et al.*, 1983). The activity of CO dehydrogenase in cell extracts of *M. thermoautotrophicum* grown on H_2/CO_2 is 200-fold less than the hydrogenase (Daniels *et al.*, 1977). However, the CO dehydrogenase activity increases fivefold and in *M. barkeri* grown on acetate is equivalent to the activity of the hydrogenase in *M. thermoautotrophicum* (Krzycki, Wolkin and Zeikus,

1982). These results support the view (Kenealy and Zeikus, 1982) that *in vivo* the function of this enzyme in acetoclastic methanogens is the catabolic decarboxylation of acetyl CoA into a methyl intermediate and a carboxyl intermediate, which is specifically dehydrogenated by CO dehydrogenase to provide electrons for methyl reduction to methane (Krzycki and Zeikus, 1984a,b). This is a reverse of the anabolic role of CO dehydrogenase during growth on one-carbon substrates. This catabolic role of CO dehydrogenase has been studied in *Methanothrix soehngii*, a methanogen capable of growing only on acetate (Kohler and Zehnder, 1984).

In the acetogenic bacteria, CO dehydrogenase appears to be fundamental to the formation of acetate and is produced constitutively (Hu, Drake and Wood, 1982; Pezacka and Wood, 1984). It has been shown that the carboxyl group of acetate formed by *C. thermoaceticum* is derived from CO, presumably from CO_2 via direct reduction by CO dehydrogenase (Hu, Drake and Wood, 1982). Acetate is then formed through the synthesis of acetyl CoA from CO, methyltetrahydrofolate and CoA.

$$^{14}CO + \text{methyltetrahydrofolate} + CoA \rightarrow 1\text{-}^{14}C \text{ acetyl-CoA} + \text{tetrahydrofolate}$$

It has been postulated that the methyltetrahydrofolate is itself synthesized from CO and tetrahydrofolate (Hu, Drake and Wood, 1982). The total synthesis of acetate from CO has been shown experimentally using glucose-limited cultures of *C. thermoaceticum* with approximately equal distribution of the ^{14}CO between the carboxyl and methyl carbons of acetic acid, when the initial gas phase was 100% CO (Martin, Misra and Drake, 1985). The pathway initially proposed for the synthesis of the methyl group involved the formation of free CO_2; this has not been demonstrated experimentally (Ragsdale *et al.*, 1983; Martin, Misra and Drake, 1985). The other possible pathway is the direct coupling of an enzyme-bound formyl-level one-carbon intermediate with the tetrahydrofolate pathway to yield 5-methyl tetrahydrofolate. However, it has been demonstrated with purified enzymes that CO dehydrogenase does not couple with the tetrahydrofolate enzymes (Ragsdale *et al.*, 1983). The apparent growth of *C. thermoaceticum* with CO as sole source of carbon and energy (Kerby and Zeikus, 1983) would require the CO to be the source of the methyl group. However, doubts have been expressed as to the validity of claiming that CO acts as sole carbon source due to the undefined nature of the medium and the presence of yeast extract (Martin, Misra and Drake, 1985).

Ragsdale and Wood (1985) have proposed that CO dehydrogenase is the central enzyme in the biosynthesis of acetate from H_2/CO_2 or CO in acetogenic bacteria. They propose four roles for the enzyme: (1) the oxidation of CO to CO_2 with the generation of reducing capacity; (2) the reduction of CO_2 to CO; (3) the formation of a one-carbon intermediate from CO; and (4) the condensation of this one-carbon intermediate with a methyl group and CoA to form acetyl CoA. This directly contradicts the hypothesis of Diekert, Hansch and Conrad (1984), based on whole-cell studies, that the

CO dehydrogenase plays only the first and second but not the third and fourth roles. This latter hypothesis fails to explain why CO dehydrogenase appears to be required as one of the four proteins for the synthesis of acetyl-CoA as described by Hu, Drake and Wood (1982).

Properties of purified carbon monoxide dehydrogenases

METHANOGENIC BACTERIA

Krzycki and Zeikus (1984b) described the purification of a CO dehydrogenase from *M. barkeri*. Most of the CO dehydrogenase activity occurred in the soluble fraction of crude cell extracts and the enzyme was purified to apparent 95% homogeneity. The molecular weight of the holoenzyme was determined by measuring ^{63}Ni elution from a gel filtration column, rather than by measuring CO dehydrogenase activity. Results of the molecular weight determinations are given in *Table 7*. An enzyme configuration of L_2S_2 was hypothesized. The absorption spectrum of this enzyme was similar to that of *A. woodii*. No hydrogenase activity was found.

Table 7. Characteristics of some purified CO dehydrogenases

Characteristic	*R. rubrum*	*M. barkeri*	*A. woodii*	*C. thermoaceticum*	
Mol.wt. of native enzyme	ND	232 000	480 000	250 000	440 000
Subunit mol. wt	ND	92 000 18 000	80 000 68 000	84 000 72 000 12 000	78 000 71 000
Ni content*	ND	ND	1·4 ± 0·1	2–3	1·7 ± 0·2
Fe/ALS content*	ND	ND	9 ± 2/11	ND	10·8 ± 1·8/11
Specific activity†	47·7	216	500	435	675
K_m for CO(mM)	0·11	5·0	ND	ND	ND
K_m for MV(mM)	0·80	ND	12·0	ND	3·03

* Content mol per mol dimeric enzyme (LS)
† Specific activities are U/mg protein, where 1U = 2 mmol MV reduced/min or 1 mmol CO oxidized/min
MV: methyl viologen; ND: not determined; ALS: acid-labile sulphide.

HOMOACETOGENIC BACTERIA

CO dehydrogenases from homoacetogens represent the most-studied forms of this enzyme. The first documented purified CO dehydrogenase from *C. thermoaceticum* was described by Drake, Hu and Wood (1980). The enzyme exhibited low CO-oxidase activity (4·31 U/mg). The absorption spectrum of the enzyme exhibited a slight shoulder at 390 nm which shifted to 415 nm during oxidation by air. Optimum activity occurred at 70°C and pH 10·6 and

in the presence of 20 mM methyl viologen. The enzyme catalysed the reduction by CO of *C. thermoaceticum* ferredoxin, *Desulfovibrio vulgaris* cytochrome c_3 and FMN.

Diekert and Ritter (1983b) purified CO dehydrogenase from *C. thermoaceticum* in the presence of CO and methyl viologen. Metal content and subunit molecular weights were determined (*Table 7*). Ragsdale *et al.* (1983) compared the CO dehydrogenases from *C. thermoaceticum* and *A. woodii*. The enzyme from *C. formicoaceticum* was too labile to purify. The metal contents of these enzymes were determined by plasma emission spectroscopy. Results obtained for Ni, Fe and Acid-Labile-Sulphide (ALS) determinations are given in *Table 7*. Magnesium, zinc and highly variable amounts of calcium (1–4 mol/mol dimer) were found. When zinc was present, magnesium was absent and vice versa. Determinations of holoenzyme and subunit molecular weights (values given in *Table 7*) suggest an $(LS)_3$ configuration for the CO dehydrogenases from *C. thermoaceticum* and *A. woodii*. However, the *A. woodii* enzyme differed in the size of the smaller subunit and its ability to exist in several active forms of molecular weight 153 000 ± 10 000 daltons bearing differing charges. The absorption spectrum of the purified enzyme from *A. woodii* is affected by redox state. In anaerobic buffer, in the absence of reducing agent, but in the presence of dithionite, the spectrum is typical of a ferredoxin-like protein, with a distinct shoulder at 390 nm. CO reduces absorbance at 390 nm and oxygen does not alter the absorption spectrum of the oxidized enzyme. The *A. woodii* enzyme also catalysed a slight formate-dependent reduction of methyl viologen. No hydrogenase activity was found.

Of several electron acceptors investigated, including flavodoxin, ferredoxin and rubredoxin isolated from *C. formicoaceticum*, rubredoxin proved the best electron acceptor of electrons from CO during catalysis by the *A. woodii* and clostridial enzymes. The fate of reduced rubredoxin is not known, but the authors reported the recent finding of the reduction of $NADP^+$ with CO by coupling ferredoxin CO dehydrogenase and a partially purified oxido-reductase fraction. Electron paramagnetic resonance (EPR) studies of *C. thermoaceticum* CO dehydrogenase indicated the presence of multiple iron–sulphur centres (Ragsdale, Ljungdahl and DerVartanian, 1982). The signals obtained were consistent with the presence of two Fe_4S_4 centres. Inactivation by oxygen rendered the enzyme EPR silent. A new signal at $g = 2{\cdot}07$ and $g = 2{\cdot}02$, formed only under anaerobic conditions in the presence of CO or HCO_3^-/CO_2, was found. The microwave power saturation behaviour and the temperature sensitivity strongly suggested that the new signal arose from a transition metal complex: this was attributed to nickel. EPR investigations of the nickel-containing hydrogenase of *M. thermoautotrophicum* gave similar results. Three lines at $g = 2{\cdot}3$, $g = 2{\cdot}2$ and $g = 2{\cdot}0$ were obtained for the nickel (III) species. However, the presence of the substrate H_2 resulted in the disappearance of the $g = 2{\cdot}3$ and $g = 2{\cdot}0$ signals. The $g = 2{\cdot}0$ radical signal was attributed to the presence of the electron acceptor benzyl viologen (Albracht, Graf and Thauer, 1982). The CO-dehydrogenase signals were postulated to be due to an interaction between Ni(III) and a carbon species formed from CO or HCO_3^-/CO_2. This was based on analogy with certain EPR characteristics of the Co(II)-carbon intermediate in ethanolamine

ammonia-lyase, a B_{12}-dependent enzyme (Baboir *et al.*, 1974). Ragsdale *et al.* (1983) using EPR on *C. thermoaceticum* and *A. woodii* CO dehydrogenase, showed that the substitution of ^{59}Ni (natural) with the ^{61}Ni isotope altered the EPR signal at $g = 2{\cdot}08$. A similar broadening of the peak at $g = 2{\cdot}02$ is found by substitution with ^{13}CO, confirming the source of these signals.

PHOTOSYNTHETIC BACTERIA

CO dehydrogenase from *R. rubrum* was purified over 600-fold (Bonam, Murrell and Ludden, 1984). The enzyme was found to be resistant to proteolytic digestion by trypsin and chymotrypsin, but the non-specific protease, subtilisin, quickly diminished activity. Some of the kinetic parameters investigated are given in *Table 7*. ^{63}Ni was found to co-migrate with CO dehydrogenase in gels, but nickel uptake from the medium was poor. Of the total CO dehydrogenase activity, 95% was found in the soluble fraction of crude cell extracts. Experiments with *R. gelatinosa* (Wakim and Uffen, 1983) showed that the partitioning of CO dehydrogenase between the soluble and membrane fractions in this organism depended on the method of preparation. Using the mildest method—osmotic lysis of sphaeroplasts—85% of the CO dehydrogenase activity remained in the membrane fraction and a membrane association of the enzyme was postulated.

Nickel component of CO dehydrogenases

The dissociation of a nickel-containing component with concomitant loss of activity was described by Drake (1982). The results obtained showed no differentiation between free Ni^{2+} or a nickel-containing protein component. A nickel-containing protein of unknown function, F_{430}, was isolated from methanogenic bacteria (Gunsalus and Wolfe, 1978) and later was shown to be a chromophore of methylreductase from *M. thermoautotrophicum* (Ellefson, Whitman and Wolfe, 1982). Evidence of the incorporation of 8-aminolevulinic acid and succinate into F_{430} suggests it is a tetrapyrrole (Diekert, Jaenchen and Thauer, 1980). Treatment of CO dehydrogenase from *C. thermoaceticum* with 0·1 M $HClO_4$ led to the release of a Ni-containing component with a molecular weight slightly larger than that of cyanocobalamin ($M_r = 1357$). Spectral studies of the native enzyme, however, suggest that this enzyme does not contain factor F_{430} (Ragsdale *et al.*, 1983).

Biotechnological applications of CO-utilizing bacteria

SINGLE-CELL PROTEIN

The synthetic gases derived from coal, lignite or peat are relatively cheap and are therefore attractive for the production of single-cell protein. They usually contain H_2 and CO and both gases may be used as energy sources for bacterial growth. The aerobic hydrogen bacteria have been considered as potential sources of single-cell protein, despite the explosive nature of the

gas and the slow growth rate of many of the bacterial strains (Schlegel and Lafferty, 1971). They may be grown on synthetic gases containing H_2 and CO, but CO inhibits growth at high concentrations (Kesler *et al.*, 1978; Savel'eva, 1979; Volova *et al.*, 1980; Cypionka and Meyer, 1982). Synthesis gas, containing roughly 50% H_2 and 50% CO, is therefore unsuitable for the growth of these organisms.

Carboxydobacteria are more suitable for the conversion of synthesis gas to single-cell protein as they can use either the hydrogen or CO components of the gas (Meyer, 1980, 1981). The growth rates of the mesophilic species are too low, but the thermophilic species have doubling times of approximately three hours on CO (*Table 5*). Stable mixtures of hydrogen bacteria and carboxydobacteria may also be used (Volova, Stasishina and Kasaeva, 1984). However, the toxic and explosive nature of synthesis gas and the poor solubility of hydrogen and carbon monoxide make it unlikely that synthesis gas will be used in this way.

ELECTROCHEMISTRY OF CO OXIDASE AND THE CONSTRUCTION OF A BIOSENSOR

Only one CO oxidase has been studied using electrochemical techniques—the enzyme from the moderate thermophile *P. thermocarboxydovorans* (Turner *et al.*, 1984, 1985). Conventional d.c. cyclic voltametry showed no indication of direct electroactivity of CO oxidase (nor indeed of the substrate CO). In the presence of CO and a mediator such as horse-heart cytochrome *c* (433 μM) or ferrocene monocarboxylic acid (200 μM), the addition of enzyme (10–100 μM) elicited a greatly enhanced anodic current and second-order rate constants of $3{\cdot}0 \times 10^4$ ℓ/mol/s and $4{\cdot}0 \times 10^5$ ℓ/mol/s respectively were determined for the reactions between cytochrome *c* or ferricinium ion and reduced enzyme at pH 7 and 20°C. This reaction was dependent on the presence of CO and indicated the sequence shown in *Figure 3*.

A CO biofuel cell was also constructed, based on a methanol fuel cell design published earlier (Turner *et al.*, 1982). The anode compartment contained 0·5 M Tris–HCl buffer, pH 7·5 and CO oxidase (2 mg) together

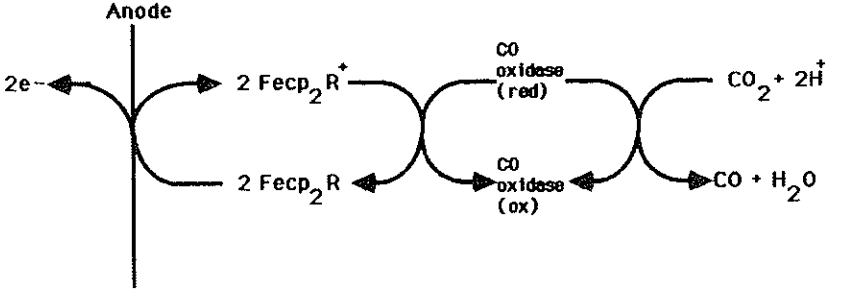

Figure 3. Electrochemistry of CO oxidase using ferrocene monocarboxylic acid as mediator. From Turner *et al.* (1984).

with either phenazine ethosulphate or methylene blue as mediator. When the anode compartment was continuously sparged with CO, the cell produced a current of approximately 1 mA for several hours. Athough responding to the addition of aliquots of CO-saturated buffer, the coulombic efficiency of the system was poor at only 14%.

Turner *et al.* (1984) also investigated several designs of CO biosensor. Initially, a homogeneous coulometric design based on that of Davis *et al.* (1983) was used. *N,N,N',N'*-tetramethyl-4-phenylenediamine was used as mediator and the platinum working electrode, responsible for reoxidizing the reduced mediator, was poised at 110 mV versus a standard cathode electrode (SCE). The charge passed was computed by integration of the current/time curve and bore a linear relationship to the amount of CO added up to a maximum of 0·35 μmol. The data obtained with this homogenous coulombic assay confirmed the two-electron stoichiometry of CO oxidation.

For all practicable purposes, a probe design of CO sensor would be required. The rapid reaction between ferricinium ion and reduced CO oxidase determined by cyclic voltametry suggested that a ferrocene mediator could be used to construct an effective enzyme electrode. The final design of probe sensor is depicted in *Figure 4*. The working electrode consists of a platinum disc coated with the virtually insoluble ferrocene derivative 1,1'-dimethylferrocene. CO oxidase is retained at the electrode surface by using a gas-permeable membrane. A cylindrical piece of silver foil was used as a pseudo-reference electrode. A potential of +150 mV versus Ag/AgCl was applied to the electrode and the amperometric response recorded. Probes responded very rapidly to CO, whether supplied in gaseous form or in

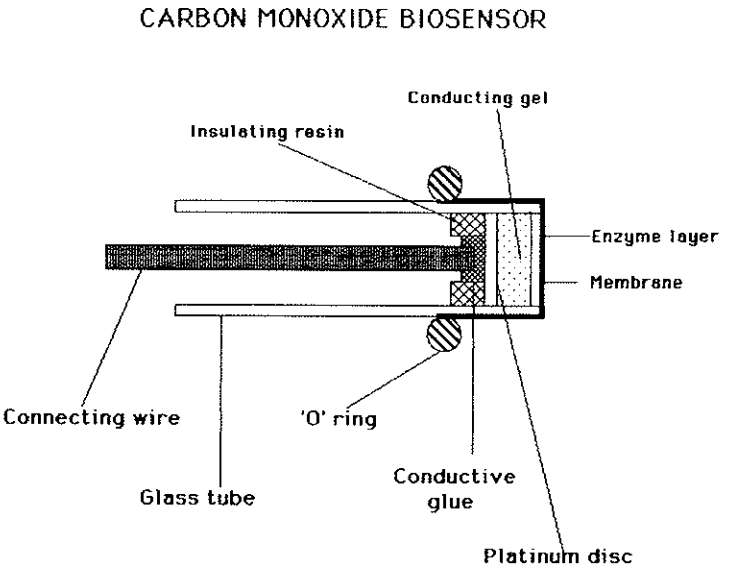

Figure 4. An enzyme-based CO-probe. From Turner *et al.* (1984).

solution, reaching a steady-state current in less than 15 s. The current obtained was directly proportional to aqueous CO concentration up to approximately 60 μM (*Figure 5*). As experimental tools the probes were relatively stable, losing only 12% of their response per hour of operation. However, this stability would need to be improved considerably before commercial application of such probes could be seriously contemplated.

ANAEROBIC BACTERIA

Microbial acetate and methane synthesis from CO or H_2/CO_2 is a biological process analogous to the Fischer–Tropsch synthesis. Nickel is known as a most efficient catalyst of acetate and methane synthesis by this industrial process (Falbe, 1982). Both acetogenesis and methanogenesis from $H_2/CO_2/CO$ gas mixtures are nickel-dependent reactions (Diekert, Graf and Thauer, 1979; Hammel *et al.*, 1984; Krzycki and Zeikus, 1984a). The nickel has been found located in the CO dehydrogenase of both acetogenic (Diekert and Thauer, 1980; Drake, Hu and Wood, 1981; Drake, 1982; Diekert and Ritter, 1983b; Ragsdale *et al.*, 1983) and methanogenic bacteria (Hammel *et al.*, 1984; Krzycki and Zeikus, 1984b). The potential of chemotrophic anaerobes for the conversion of synthesis and other gasifier gases has been recognized, and some studies carried out. The anaerobic species isolated which can grow on these gas mixtures ($H_2/CO_2/CO$ in various ratios) are particularly attractive because of their high thermodynamic efficiencies of cell synthesis, about 55–60% (Zeikus, 1983), and their natural end-products, for example acetic and butyric acids.

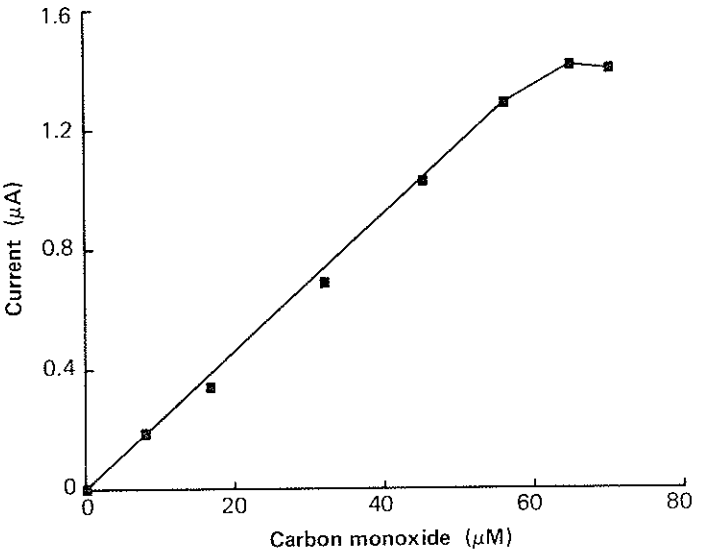

Figure 5. Calibration curve for an enzyme-based CO-sensor. CO was added as aliquots of CO-saturated buffer and the steady-state output of the sensor recorded.

Methanogens have performed a pivotal role in anaerobic digestions for over a century. However their role has been perceived as primarily the removal of waste and not the generation of fuel. This could be altered by channelling carbon flow away from sulphidogenesis and enhancing the conversion of acetate to methane. Modulation either biochemically or genetically of the activity of the CO dehydrogenase could be effective to this end. Biomethanation is defined as the anaerobic fermentation of CO, CO_2 and H_2 to methane. This has been studied with particular reference to the replacement of petroleum-based chemicals and fuels. Primarily, this has involved the conversion of H_2/CO_2 to methane, although by using known CO-oxidizers, *Rhodopseudomonas* spp. (Uffen, 1975, 1976), it is possible to convert the CO component of a gas mixture to CO_2 and then to methane (Wise, Cooney and Angenstein, 1978). Biomethanation may also be used to upgrade the thermal (BTU) content of gasification mixtures, and to remove toxic CO via the biocatalytic application of methanogens in high-pressure reactions (Wise, Cooney and Angenstein, 1978).

Homoacetogens have more apparent biocatalytic applications than methanogens. *B. methylotrophicum* (CO strain) has been grown on CO alone in fed-batch culture yielding acetate and small amounts of butyrate, with a yield of about 75% (Datta, 1982). There is a problem with recovery of these organic acids; one novel products-recovery method, applied to cultures of *B. methylotrophicum*, esterified the fermentation liquor yielding methylbutyrate and enhancing end-product formation. Methylbutyrate is a chemical feedstock for plastics (Zeikus, 1983). Other studies have focused on the use of mixed cultures and mixtures of gases, CO_2/H_2 and CO/H_2. In these the main products were acetic through caproic acids with traces of even-numbered acids (Levy *et al.*, 1981). These studies were based on the isolation of mixed cultures from various natural sources. It has been suggested that a more fruitful approach would be the immobilization of mixtures of strains known to perform desirable conversions, or to enrich for specific product formation rather than just gas utilization (Daniels, 1982). This is the only example of direct selection for CO utilization, indicating that anaerobic CO-utilizers do exist in the natural state.

A wider range of products could be made available by regulatory or genetic manipulation. One possible regulatory manipulation involves the use of low levels of CO (dissolved CO corresponding to a CO partial pressure of 0·1–0·2 atmospheres; 10·1–20·2 kPa) to modulate acetone–butanol–ethanol fermentation by *Clostridium acetobutyricum*. The CO acts by specifically inhibiting hydrogenases, leading to an increase in the pool of reduced nucleotides and an increase in the products requiring reduced nucleotides for their formation, namely butanol and ethanol (Datta and Zeikus, 1985).

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